University of Alberta

Molecular characterization of *Lactobacillus gallinarum* isolates

from the broiler chicken crop

by

Karen Elaine Hagen

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Abstract

The crop microflora of boiler chickens was investigated using PCR-Denaturing Gradient Gel Electrophoresis and lactobacilli were cultivated. *Lactobacillus* isolates were speciated, and *L. gallinarum* was found to be present in the crops of different chickens. Genetic fingerprinting of *L. gallinarum* isolates revealed 17 strains were represented among 44 isolates. Cloning and sequencing of surface protein (S-protein) genes from the isolates revealed at least 7 genes. One gene was found in most *L. gallinarum* isolates and may be necessary for persistence in the gastrointestinal tract. A second gene was isolated from 2 strains and also from the type strain ATCC 33199 (a chicken crop isolate). Therefore, this research revealed two different S-protein genes are conserved among crop *L. gallinarum*. Future work could investigate the role of these proteins in mediating adherence of *L. gallinarum* isolates to the chicken crop.

Dedication

Dedicated to my husband, Dan Hagen, for his patience and understanding. For hanging out with me while I wrote this thesis, and also for writing the note below one Sunday evening, which cheered me up immensely.

> "Who? Well ME of course, your alter-ego. Katharine the Conqueror, Ruler of Nations and of Molecular Biology, bending and coaxing the powers to your will. For it was in an age ancient, time only in it's infancy, that you deliberated the blood of which all matter births. It was yours to deal as you wish, and so you did, with a but a tender blow you downed kingdoms and nations."

Acknowledgement

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I would like to thank Dr. Todd Klaenhammer (North Carolina State University) and Dr. Lynn McMullen (University of Alberta) for the provision of reference cultures, and Matt Rawluk and Dorthe Nielsen for their assistance with sampling and plating. I would also like to thank Teresa Grayson for the production of competent cells and for assisting in the identification of isolates, and Bernadette Steman for DNA sequencing. The assistance of the animal research technicians at the Alberta Poultry Research Centre, University of Alberta, and Renate Meuser of the Agriculture, Food, and Nutritional Science Biotechnology Centre was greatly appreciated.

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List of Symbols, Nomenclature, or Abbreviations

° degrees

% percent

~ approximately

°C Degrees Celsius

16S rDNA 16S ribosomal RNA gene

16-23S rDNA 16S rDNA (partial) + ITS + 23S rDNA (partial)

23S rDNA 23S ribosomal RNA gene

2YT yeast tryptone

A (in DNA sequence) adenosine

aa amino acid

Ag/For Agriculture Forestry Centre

ABLN Alcian Blue-Lanthanum Nitrate

ApaI *Acetobacter pasteurianus* sub. *pasteurianus* restriction enzyme I

ARDRA amplified ribosomal DNA restriction analysis

ATCC American Type Culture Collection

ATP adenosine triphosphate

BCE before common era

BD Becton Dickinson

BLAST basic local alignment search tool

bp base pairs

BSA bovine serum albumin

BSH bile salt Hydrolase

C (in DNA sequence) cytosine

ca. *circa*

Cat. catalogue

cbs collagen binding substance gene

Cbs collagen binding substance

CFU colony forming units

CHEF clamped homogeneous electrical field

Cm chloramphenicol

cm centimetres

c-myc human proto oncogene

CNRZ Centre INRA de Jouy-en-Josas

CWB cell wash buffer

CWF cell wall fraction

DGGE denaturing gradient gel electrophoresis

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

$\mathbf{D_{sc}}$ Dice's similarity coefficient

DTT dithiothreitol

E. *Escherichia*

ECM extracellular matrix

***Eco*RI** *Escherichia coli* restriction enzyme I

EDTA ethylene diamine tetra acetic acid

EPS extracellular polysaccharide

EtBr Ethidium Bromide

ETEC enterotoxigenic *Escherichia coli*

Fig. figure

FIGE field inversion gel electrophoresis

G (in DNA sequence) guanine

g grams

GAA group A *acidophilus*

GFP green fluorescent protein

GIT gastrointestinal tract

GTE glucose Tris EDTA

***gus*A** β-glucuronidase gene

***Hae*III** *Haemophilus aegyptius* restriction enzyme III

HGFP His tag/GFP

His histidine

hr hour

hrs hours

Inc incorporated

IPTG isopropyl-beta-D-thiogalactopyranoside

ITS intergenic transcribed sequence

JCM Japan Collection of Microorganisms

K (in DNA sequence) T or G

kb kilobases

kDa kiloDaltons

L litres

L. *Lactobacillus*

Lc. *Lactococcus*

LAB lactic acid bacteria

LB Luria Bertani

LBS *Lactobacillus* selective

LBS+TJ *Lactobacillus* selective and clarified tomato juice

LDH lactate dehydrogenase

LMG Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection

LTA lipoteichoic acid

luc luciferase gene

M molar

mg milligrams

min minutes

mL millilitres

mm millimetre

mM millimolar

mol% GC percent GC content

mRNA messenger RNA

MRS deMann, Rogosa, Sharpe

***Mse*I** *Micrococcus* species restriction enzyme I

N (in DNA sequence) unknown nucleotide

N/A not applicable

ND note done

ng nanograms

nM nanomolar

nm nanometres

no. number

nt nucleotides

OD optical density

OD_{550} optical density at 550 nm

OD_{600} optical density at 600 nm

ORF open reading frame

P promoter

PCR polymerase chain reaction

***pep*N** aminopeptidase N gene

PFGE pulse field gel electrophoresis

pGEMT plasmid GEMT

pI isoelectric point

pmol picomol

***prt*P** proteinase P gene

RAPD random amplified polymorphic DNA

RNA ribonucleic acid

RNase A ribonucelase A

rpm revolutions per minute

RT-PCR reverse transcriptase PCR

SAC S-protein C terminus

SAN S-protein N terminus

SDS sodium dodecyl sulphate

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

sec seconds

SLH s-layer homologous

slp/slpn S-layer protein gene

Slp/Slpn S-layer protein

***Sma*I** *Serratia marcescens* Sb restriction enzyme I

S-protein surface protein

SSC standard sodium citrate

SSPE sodium SDS phosphate EDTA

T (in DNA sequence) thymine

TA teichoic acid

Taq *Thermus aquaticus*

TBE Tris boric acid EDTA

TE Tris EDTA

TEM transmission electron microscopy

TEMED N,N,N',N'-Tetramethylethylenediamine

TGGE temperature gradient gel electrophoresis

TTGE temporal temperature gradient electrophoresis

U activity units

V volts

v/v volume per volume

W (in DNA sequence) A or T

w/v weight per volume

xg relative centrifugal force (rcf)

x-Gal 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

µg micrograms

µL microlitres

Y (in DNA sequence) C or T

Chapter 1: *Lactobacillus* and the Chicken Gastrointestinal Tract

1.1. The *Lactobacillus* spp.: taxonomy and natural environments

1.1.1: Overview

Lactobacilli are Gram-positive rod shaped nutritionally fastidious bacteria [88]. Some lactobacilli are members of the microflora of humans, animals, and birds [66], while others are commonly associated with plants, soil, water, sewage and cereal products, and are used to make fermented products such as cheese, fermented meats, wine and beer, sourdough bread and silage [88]. Lactobacilli are members of a larger group of fermentative bacteria referred to as the Lactic Acid Bacteria (LAB). LAB are nutritionally fastidious, Gram-positive aerotolerant anaerobic bacteria that do not form spores [94], contain low guanine and cytosine (mol% GC) content in their DNA (33-55 mol% GC for lactobacilli) [77] and include the following genera [88]: *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, *Weissella*, *Carnobacterium*, *Pediococcus*, *Streptococcus* and *Lactobacillus*. The production of lactic acid as the main fermentation product is the criteria used to define members of the LAB. LAB fermentation patterns may include only one end product (lactic acid) in homofermentative LAB or multiple end products (such as ethanol, CO_2, acetate, formate, succinate) in heterofermentative LAB [77]. Most LAB can consequently tolerate low pH [94]. LAB were an integral part of the first bacteriological studies; some of the bacteria studied by Pasteur and Lister were likely LAB and the first pure culture studied by Lister was *Lactococcus lactis* [94]. LAB also played an important role in the history of

molecular biology and genetics; Griffith [37] and Avery, McCarthy and MacLeod [4] studied variants of *Streptococcus* [94].

1.1.2: Taxonomy of the lactobacilli

LAB taxonomy has undergone a number of revisions since the initial discovery and classification of LAB species. Orla-Jensen [72] developed the following biochemical properties to group and identify member species (now known as "traditional" speciation methods): the ability to grow at 15°C or in the presence of oxygen, motility, the isomers of lactic acid produced in fermentation, the production of ammonia from arginine, tolerance to varying concentrations of salt, and the ability to ferment a variety of carbohydrates [66,94]. Recent molecular analysis, reviewed by Schleifer [77,78] has modified the initial classifications and in some cases, separated biochemically similar species into a number of related species or subspecies. Modern molecular techniques for identification have utilized the species specific sequence of 16S and/or 23S ribosomal ribonucleic acid (rRNA) genes (rDNA) as well as those for several other genes, although 16S rDNA sequence is universally considered the "gold standard" for species identification [77]. Fig. 1.1 shows a representative phylogenetic tree based on 16S rDNA sequence of lactobacilli and related LAB.

The *Lactobacillus* genus and closely related genera were divided into three groups based on 16S rDNA sequence: Group I containing *L. delbrueckii* and *L. acidophilus*; Group II containing many *Lactobacillus* species including *L. aviarius*, *L. salivarius*, *L. reuteri* and *L. fermentum* as well as all 6 species of *Pediococcus*; and Group III containing all *Leuconostoc* species and other lactobacilli [78]. However, other classifications based on both molecular techniques and advanced biochemical analyses

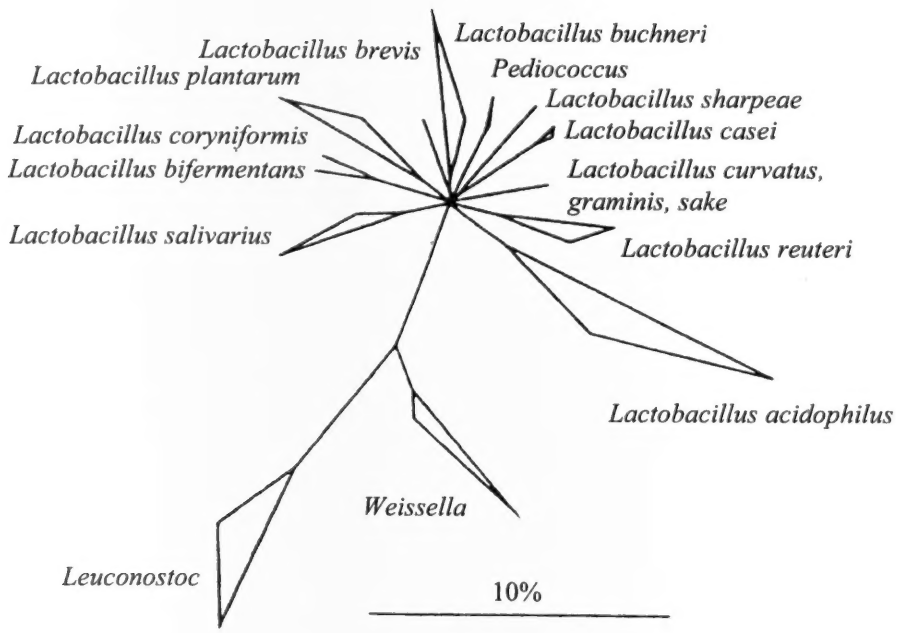

Fig. 1.1. Phylogenetic tree of lactobacilli and related genera based on analysis of 16S rDNA sequence. Adapted from Schleifer et al. [78].

have resulted in different and more detailed groupings of only the lactobacilli. Based on this classification, the lactobacilli can be alternatively grouped. Group 1, obligate homofermenters, containing the *L. acidophilus* group (reviewed below), *L. aviarius*, *L. salivarius* and *L. helveticus*; Group 2, facultative heterofermenters, including *L. casei*, *L. paracasei* and *L. rhamnosus*; and Group 3, obligate heterofermenters, containing *L. brevis*, *L. reuteri* and *L. fermentum* [52,88]. *L. helveticus* is closely related but taxonomically distinct from the *L. acidophilus* group [88]. Therefore, lactobacilli reduce the pH of their environments and consequently inhibit acid sensitive bacteria.

The *L. acidophilus* group are members of the gastrointestinal microflora of humans [66], pigs [6] and chickens [26,27,95]. In the 1980s, Johnson et al. [44] and Lauer et al. [59] discovered heterogeneity among *L. acidophilus* strains based on DNA-DNA homology. Johnson et al. [44] divided *L. acidophilus* into two DNA homology groups. Group A (group A *acidophilus* or GAA) consists of 4 highly related species (*L. acidophilus*, *L. amylovorus*, *L. crispatus* and *L. gallinarum*) and group B consists of *L. gasseri* and *L. johnsonii*. Due to the relatedness of the *L. acidophilus* group, it is difficult to distinguish among its members using traditional biochemical techniques (as used in older literature) and even using molecular techniques (for example, compare 16S rDNA similarity among *L. acidophilus* group members in Fig. 1.2*) Therefore, species designations from older literature must be regarded with some caution [66], and only very precise molecular speciation methods used in current literature can fully

---

* Alignments for 16S rDNA and S-protein/S-protein gene sequences were performed with ClustalW using either the MegAlign module of the Lasergene software package (DNAStar Inc Madison, WI) or the ClustalW online alignment tool (http://www.ebi.ac.uk/clustalw/) with the default settings.

A.

```
L. acidophilus ATCC 4356   ----------------------------------------------------
L. amylovorus ATCC 33620   ----------------------------------------------------
L. crispatus ATCC 33820    ----------------------------------------------------
L. gallinarum ATCC 33199   ----------------------------------------------------
L. gasseri ATCC 33323      CAAGCAATAAATTTGAGATAACTCAAAGAAAGTTTTAGAGCTAAACGATA 50
L. johnsonii ATCC 33200   ----------------------------------------------------

L. acidophilus ATCC 4356   -------------------NNAAAACGAGAGTTTGATCCTGGCTCAGGAC 31
L. amylovorus ATCC 33620   -----------------NNNTANAATGAGAGTTTGATCCTGGCTCAGGAC 33
L. crispatus ATCC 33820   ---------------------------AGAGTTTGATNNTGGCTCAGGAC 23
L. gallinarum ATCC 33199  ------------------------------------CTGGCTCAGGAC 12
L. gasseri ATCC 33323      AAAAGCTCATTTTCAAGAAGGAAAATGAGAGTTTGATCCTGGCTCAGGAC 100
L. johnsonii ATCC 33200  ----------------------------------------------------

L. acidophilus ATCC 4356   GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAAC 81
L. amylovorus ATCC 33620  GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAAC 83
L. crispatus ATCC 33820  GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACTAAC 73
L. gallinarum ATCC 33199 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCAGAACCAGC 62
L. gasseri ATCC 33323    GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGA 150
L. johnsonii ATCC 33200  -------GGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGA 43
                           ************************************    * *

L. acidophilus ATCC 4356  AGATTC----ACTTCGGT--GATGACGTTGGGNAAC-GCTAGCGGCGGAT 124
L. amylovorus ATCC 33620  AGATTT----ACTTCGGT--AATGACGTTGNAAA----CNAGCGGCGGAT 123
L. crispatus ATCC 33820  AGATTT----ACTTCGGT--AATGACGTTAGGAAA--GCGAGCGGCGGAT 115
L. gallinarum ATCC 33199  AGATTT----ACTTCGGT--AATGACGCTGGGGAC--GCGAGCGGCGGAT 104
L. gasseri ATCC 33323     TGAATTTGGTGCTTGCACCAGATGAAACTAGATACAAGCGAGCGGCGGAC 200
L. johnsonii ATCC 33200  TGATTTTAGTGCTTGCACTAAATGAAACTAGATACAAGCGAGCGGCGGAC 93
                            ** *      ***       ****   *    *    * *********

L. acidophilus ATCC 4356  GGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGG 174
L. amylovorus ATCC 33620  GGGTGAGTAACACGTGGGGAACCTGCCCCNAAGTCTGGGATACCATTTGG 173
L. crispatus ATCC 33820  GGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGG 165
L. gallinarum ATCC 33199  GGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGG 154
L. gasseri ATCC 33323     GGGTGAGTAACACGTGGGTAACCTGCCCAAGAGACTGGGATAACACCTGG 250
L. johnsonii ATCC 33200  GGGTGAGTAACACGTGGGTAACCTGCCCAAGAGACTGGGATAACACCTGG 143
                           ****************** *********   ** ******** **  ***

L. acidophilus ATCC 4356  AAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATA 224
L. amylovorus ATCC 33620  AAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTG 223
L. crispatus ATCC 33820  AAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTTTN 215
L. gallinarum ATCC 33199  AAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATA 204
L. gasseri ATCC 33323     AAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTA 300
L. johnsonii ATCC 33200  AAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTG 193
                           ****** ***************** **  *    ******   **  * *
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Fig. 1.2. Comparison of 16S rDNA sequence from the type strains of species of the *L. acidophilus* group. **A.** ClustalW alignment. "*" indicates identical nucleotide in all sequences. The GenBank accession numbers are as follows: ATCC 4356, LBARR16SA; ATCC 33620, LBARR16SD; ATCC 33820, LCR17362; ATCC 33199, LGA417737; ATCC 33323, LGA242968; ATCC 33200, LBARR16SAZ. **B.** Phylogenetic tree based on DNA sequence comparisons. **C.** Percent identity between DNA sequences.

A.

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L. acidophilus ATCC 4356    AAAGGCGGCGTAAGCTGTCGCTATGGNNTGGCCCCGCGGTGCATTAGCTA 274
L. amylovorus ATCC 33620   AAAGGCGGCGTAAGCTGTCGCTNAGGGATGGCCCNGCGGTGCATTAGCTA 273
L. crispatus ATCC 33820    AAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTA 265
L. gallinarum ATCC 33199   AAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTA 254
L. gasseri ATCC 33323      AAAGATGGT-TCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTA 349
L. johnsonii ATCC 33200    AAAGATGGT-TCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTA 242
                            ****  **  *  *** ** **   *  *** ** **************

L. acidophilus ATCC 4356    GTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGA 324
L. amylovorus ATCC 33620   GTTGGTAAGGTAACGGCTTACCAAGGCGACGATGCATAGCCGAGTTGAGA 323
L. crispatus ATCC 33820    GTTGGTAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGA 315
L. gallinarum ATCC 33199   GTTGGTAAGGTAACGGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGA 304
L. gasseri ATCC 33323      GTTGGTAAGGTAACGGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGA 399
L. johnsonii ATCC 33200    GTTGGTAAGGTAATGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGA 292
                            ******* ***** *** ********* * ********************

L. acidophilus ATCC 4356    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG 374
L. amylovorus ATCC 33620   GACTGATCGGCCACATTGGGACTGAGACACGGCCCNAACTCCTACGGGAG 373
L. crispatus ATCC 33820    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG 365
L. gallinarum ATCC 33199   GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG 354
L. gasseri ATCC 33323      GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG 449
L. johnsonii ATCC 33200    GACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG 342
                            ************************************ **************

L. acidophilus ATCC 4356    GCAGCNGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGC 424
L. amylovorus ATCC 33620   GCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGC 423
L. crispatus ATCC 33820    GCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGC 415
L. gallinarum ATCC 33199   GCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGC 404
L. gasseri ATCC 33323      GCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGC 499
L. johnsonii ATCC 33200    GCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGC 392
                            ***** ************************ ********************

L. acidophilus ATCC 4356    CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAG 474
L. amylovorus ATCC 33620   CGCNTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAG 473
L. crispatus ATCC 33820    CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAG 465
L. gallinarum ATCC 33199   CGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAG 454
L. gasseri ATCC 33323      CGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTAGTGAAG 549
L. johnsonii ATCC 33200    CGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTAGTGAAG 442
                            *** ************** ******* ***************** * *******

L. acidophilus ATCC 4356    AAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAG 524
L. amylovorus ATCC 33620   AAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAG 523
L. crispatus ATCC 33820    AAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAG 515
L. gallinarum ATCC 33199   AAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAG 504
L. gasseri ATCC 33323      AAAGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATTACTTAGAAAG 599
L. johnsonii ATCC 33200    AAAGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATTACTTAGAAAG 492
                            ** ************************************ *   *******

L. acidophilus ATCC 4356    TCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCNAGCG 574
L. amylovorus ATCC 33620   TCTCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCNAGCG 573
L. crispatus ATCC 33820    TCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG 565
L. gallinarum ATCC 33199   TCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG 554
L. gasseri ATCC 33323      TCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG 649
L. johnsonii ATCC 33200    TCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG 542
                            ** ****************************************** ****

L. acidophilus ATCC 4356    TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCT 624
L. amylovorus ATCC 33620   TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAAAATAAGTCT 623
L. crispatus ATCC 33820    TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCT 615
L. gallinarum ATCC 33199   TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCT 604
L. gasseri ATCC 33323      TTGTCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGTTCAATAAGTCT 699
L. johnsonii ATCC 33200    TTGTCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGTTCAATAAGTCT 592
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A.

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L. acidophilus ATCC 4356   GATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTT 674
L. amylovorus ATCC 33620  NATGTGAAAGCCCTCGGCTTNACCGAGNNACTGCATCGGAAACTGTTTTT 673
L. crispatus ATCC 33820  GATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTT 665
L. gallinarum ATCC 33199 GATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTT 654
L. gasseri ATCC 33323   GATGTGAAAGCCTTCGGCTCAACCGGAGAATTGCATCAGAAACTGTTGAA 749
L. johnsonii ATCC 33200 GATGTGAAACGCTTCGGCTCAACCGGAGAATTGCATCAGAAACTGTTGAA 642
                           ******** * ******  ****    * ******* *********

L. acidophilus ATCC 4356   CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 724
L. amylovorus ATCC 33620  CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 723
L. crispatus ATCC 33820  CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 715
L. gallinarum ATCC 33199 CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 704
L. gasseri ATCC 33323   CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 799
L. johnsonii ATCC 33200 CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCG 692
                           ************************************************

L. acidophilus ATCC 4356   TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAAC 774
L. amylovorus ATCC 33620  TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCNCTCTGGTCTGCAAC 773
L. crispatus ATCC 33820  TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAAC 765
L. gallinarum ATCC 33199 TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAAC 754
L. gasseri ATCC 33323   TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAAC 849
L. johnsonii ATCC 33200 TAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAAC 742
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L. acidophilus ATCC 4356   TGACGCTGAGGCTCNNAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 824
L. amylovorus ATCC 33620  TGACGCTGAGGCTCGNAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 823
L. crispatus ATCC 33820  TGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 815
L. gallinarum ATCC 33199 TGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 804
L. gasseri ATCC 33323   TGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 899
L. johnsonii ATCC 33200 TGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGG 792
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L. acidophilus ATCC 4356   TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTC 874
L. amylovorus ATCC 33620  TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTC 873
L. crispatus ATCC 33820  TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTC 865
L. gallinarum ATCC 33199 TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCTTC 854
L. gasseri ATCC 33323   TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTC 949
L. johnsonii ATCC 33200 TAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTC 842
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L. acidophilus ATCC 4356   TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 924
L. amylovorus ATCC 33620  TCAGTGCTGCAGCTAACGCATTAAGCTCTCCGCCTGGGGAGTACGACCGC 923
L. crispatus ATCC 33820  TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 915
L. gallinarum ATCC 33199 TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 904
L. gasseri ATCC 33323   TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 999
L. johnsonii ATCC 33200 TCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGC 892
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L. acidophilus ATCC 4356   AAGGTTGAAACTCAAAGGAATTGACGGGGNCCCGCACAAGCGGTGGAGCA 974
L. amylovorus ATCC 33620  AAGGTTGAAACTCAAAGGAATTGACGGGGGCCNGCACAAGCGGTGGAGCA 973
L. crispatus ATCC 33820  AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA 965
L. gallinarum ATCC 33199 AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA 954
L. gasseri ATCC 33323   AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA 1049
L. johnsonii ATCC 33200 AAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA 942
                           ****************************** ** ****************

L. acidophilus ATCC 4356   TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCT 1024
L. amylovorus ATCC 33620  TGTGGTTTAATTCGAANNNACGCGAAGAACCTTACCAGGNCTTGACATCT 1023
L. crispatus ATCC 33820  TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCT 1015
L. gallinarum ATCC 33199 TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCT 1004
L. gasseri ATCC 33323   TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC 1099
L. johnsonii ATCC 33200 TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC 992
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A.

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L. acidophilus ATCC 4356    AGTGCAATCCGTAGAGATACGGNGTTCCCTTCGGGGACACTAAGACAGGT 1074
L. amylovorus ATCC 33620    AGTGCNATCTGTAGAGATATGGAGTTCCCTTCGGGGNCGCTAAGACAGGT 1073
L. crispatus ATCC 33820    AGTGCCATTTGTAGAGATACAAAGTTCCCTTCGGGGACGCTAAGACAGGT 1065
L. gallinarum ATCC 33199    AGTGCCATCCTAAGAGATTAGGAGTTCCCTTCGGGGACGCTAAGACAGGT 1054
L. gasseri ATCC 33323    AGTGCAAACCTAAGAGATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGT 1149
L. johnsonii ATCC 33200    AGTGCAAACCTAAGAGATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGT 1042
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L. acidophilus ATCC 4356    GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1124
L. amylovorus ATCC 33620    GGNGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1123
L. crispatus ATCC 33820    GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1115
L. gallinarum ATCC 33199    GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1104
L. gasseri ATCC 33323    GGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG 1199
L. johnsonii ATCC 33200    GGTGCATGGCTGTCGTCAGCTCGTGTCATGAGATGTTGGGTTAAGTCCCG 1092
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L. acidophilus ATCC 4356    CAACGAGTGCAACCCTTGTCATTAGTTGCCAGCATTAAGTTGGGCACTCT 1174
L. amylovorus ATCC 33620    CAACGAGCGCAACCCTTGTTATTAGTTGCCAGCATTAAGTTGGGCACTCT 1173
L. crispatus ATCC 33820    CAACGAGCGCAACCCTTGTTATTAGTTGCCAGCATTAAGTTGGGCACTCT 1165
L. gallinarum ATCC 33199    CAACGAGCGCAACCCTTGTTATTAGTTGCCAGCATTAAGTTGGGCACTCT 1154
L. gasseri ATCC 33323    CAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCT 1249
L. johnsonii ATCC 33200    CAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCT 1142
                            ******* *********** *********** ***************

L. acidophilus ATCC 4356    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1224
L. amylovorus ATCC 33620    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1223
L. crispatus ATCC 33820    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1215
L. gallinarum ATCC 33199    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1204
L. gasseri ATCC 33323    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1299
L. johnsonii ATCC 33200    AATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC 1192
                            **************************************************

L. acidophilus ATCC 4356    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGTACA 1274
L. amylovorus ATCC 33620    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGTACA 1273
L. crispatus ATCC 33820    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGTACA 1265
L. gallinarum ATCC 33199    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGTACA 1254
L. gasseri ATCC 33323    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACA 1349
L. johnsonii ATCC 33200    ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACA 1242
                            ********************************************* * *****

L. acidophilus ATCC 4356    ACGAGGAGCAAGCCTGCGAAGGCAAGCGAATCTCTTAAAGCTGTTCTCAG 1324
L. amylovorus ATCC 33620    ACGAGAAGCAAGCCTGCGAAGGCNAGCGAATCTCTGAAAGCTGTTCTCAG 1323
L. crispatus ATCC 33820    ACGAGAAGCGAGCCTGCGAAGGCAAGCGAATCTCTGAAAGCTGTTCTCAG 1315
L. gallinarum ATCC 33199    ACGAGAAGCGAGCCTGCGAAGGCAAGCGAATCTCTGAAAGCTGTTCTCAG 1304
L. gasseri ATCC 33323    ACGAGAAGCGAACCTGCGAAGGCAAGCGGATCTCTGAAAGCCGTTCTCAG 1399
L. johnsonii ATCC 33200    ACGAGAAGCGAACCTGCGAAGGCAAGCGGATCTCTTAAAGCCGTTCTCAG 1292
                            ***** *** * ************ **** ****** ***** ********

L. acidophilus ATCC 4356    TTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAA 1374
L. amylovorus ATCC 33620    TTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAA 1373
L. crispatus ATCC 33820    TTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAA 1365
L. gallinarum ATCC 33199    TTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAA 1354
L. gasseri ATCC 33323    TTCGGACTGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAA 1449
L. johnsonii ATCC 33200    TTCGGACTGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAA 1342
                            ********* ** ********** ** ************************

L. acidophilus ATCC 4356    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1424
L. amylovorus ATCC 33620    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCNTTGTACACACC 1423
L. crispatus ATCC 33820    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1415
L. gallinarum ATCC 33199    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1404
L. gasseri ATCC 33323    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1499
L. johnsonii ATCC 33200    TCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1392
                            ************************************** ***********
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A.

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L. acidophilus ATCC 4356    GCCCGTCACACCATGGGAGTCTGCAATGCCCAAAGCCGGTGGCCTAACCT 1474
L. amylovorus ATCC 33620    GCCCGTCACACCATGGGAGTCTGCAATGCCCAAAGCCGGTGGCCTNACNN 1473
L. crispatus ATCC 33820     GCCCGTCACACCATGGGAGTCTGCAATGCCCAAAGCCGGTGGCCTAACCT 1465
L. gallinarum ATCC 33199    GCCCGTCACACCATGGAAGTCTGCAATGCCCAAAGCCGGTGGCCTAACCT 1454
L. gasseri ATCC 33323       GCCCGTCACACCATGAGAGTCTGTAACACCCAAAGCCGGTGGGATAACCT 1549
L. johnsonii ATCC 33200     GCCCGTCACACCATGAGAGTCTGTAACACCCAAAGCCGGTGGGATAACCT 1442
                            ***************  ****** **  ************** * **

L. acidophilus ATCC 4356    TCG--GGAAGGAGCCGTCTAAGGCAGGGCAGATGACNNNNNNNNNNNNGT 1522
L. amylovorus ATCC 33620    TCG--GGAAGGAGCCGTCTAAGGCAGGGCAGATGACNGGGG--------- 1512
L. crispatus ATCC 33820     TCG--GGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGT 1513
L. gallinarum ATCC 33199    TCG--GGAAGGAGCCGTCTAAGGCAGGGCAGATGACTGGGGTGAAGTCGT 1502
L. gasseri ATCC 33323       TTATAGGAGTCAGCCGTCTAAGGTAGGACAGATGATTAGGGTGAAGTCGT 1599
L. johnsonii ATCC 33200     TTATAGGAGTCAGCCGTCTAAGGTAGGACAGATGATTAGGGTGAA----- 1487
                            *    ***   ************ *** *******

L. acidophilus ATCC 4356    AACAAGNNNNNNNNNNNNGAACCTGNNNNNNGATCACCTCCTTTCTA 1569
L. amylovorus ATCC 33620    ------------------------------------------------
L. crispatus ATCC 33820     AACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCTTTNN- 1559
L. gallinarum ATCC 33199    AACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCTTT--- 1546
L. gasseri ATCC 33323       AACAAGGTAGCCGTAGGAGAACCTGCGGCTGGATCACCTCCTTT--- 1643
L. johnsonii ATCC 33200     ------------------------------------------------
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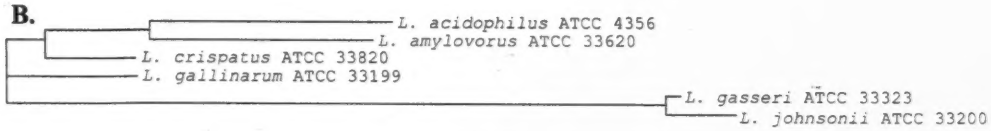
B.


L. acidophilus ATCC 4356
L. amylovorus ATCC 33620
L. crispatus ATCC 33820
L. gallinarum ATCC 33199
L. gasseri ATCC 33323
L. johnsonii ATCC 33200

C.

	L. acidophilus ATCC 4356	*L. amylovorus* ATCC 33620	*L. crispatus* ATCC 33820	*L. gallinarum* ATCC 33199	*L. gasseri* ATCC 33323	*L. johnsonii* ATCC 33200
L. acidophilus ATCC 4356		95	95	95	89	90
L. amylovorus ATCC 33620			96	95	90	89
L. crispatus ATCC 33820				98	92	91
L. gallinarum ATCC 33199					93	91
L. gasseri ATCC 33323						99
L. johnsonii ATCC 33200						

distinguish among *L. acidophilus* group members. Despite their demonstrated presence as members of the gastrointestinal tract (GIT) microflora, the mechanisms by which the GAA survive the harsh environment encountered in the GIT are not fully known. In particular, *L. amylovorus* and *L. gallinarum* are poorly studied and little is known about them.

1.2. Microbial ecology of the chicken gastrointestinal tract

1.2.1: Overview

While bacteria can be cultivated from any site in the gastrointestinal tract, only certain areas are considered to have a "microflora" [93]. These areas can vary somewhat from host to host; in the chicken, the regions of colonisation include the crop, ileum, caeca and colon [28,93]. The avian gastrointestinal tract is adapted to assist birds in flight by containing a shorter intestine than other animals and the presence of a beak rather than teeth and jaw muscles [22]. Further, birds can swallow their food whole and digest it later due to the combined functions of the oesophagus, crop and gizzard. The oesophagus and crop are highly distensible to allow the passage and storage, respectively of whole food. Chickens do not produce salivary amylase, although amylase activity may come from other sources, including bacteria. With respect to nutritional effects, the crop is not necessary for normal growth and development in *ad libitum* fed birds as its physiological role is for collection of food for later digestion. However, the crop microflora may have important effects on other areas of the gastrointestinal tract and may exclude pathogens or otherwise undesirable bacteria from the GIT, as discussed below. The proventriculus is a secretory stomach where acid and digestive enzymes are secreted. Food is then moved into the large and well-muscled gizzard, where it is physically and chemically digested.

The gizzard is lined with a cuticle to protect it from acid, proteolytic enzymes, and abrasive foods such as grains. The small intestine consists of the duodenum, jejunum, and ileum. The ileum is a site of carbohydrate, amino acid, and fatty acid uptake. The paired caeca contain anaerobic bacteria that ferment undigested material [64]; carbohydrates, amino acids and water are also absorbed [22]. The caeca empty into the short colon for elimination through the cloaca, which also connects to the reproductive and urinary tracts. [22].

1.2.2: *Lactobacillus* found in chicken gastrointestinal tract

It is a well-established fact that lactobacilli exist in the gastrointestinal tract of chickens and can predominate in specialized organs (i.e. the crop). A wealth of early literature used culture-dependent techniques to isolate and identify gastrointestinal bacteria, and in some cases, yeasts [87]. However, it should be noted that limitations in the ability to culture anaerobic bacteria undoubtedly had an effect on the types of bacteria isolated from the gastrointestinal tract of chickens, although certain techniques could be employed to improve the recovery of otherwise uncultivatable bacteria (e.g. [5]). In general, the most common species of *Lactobacillus* isolated from the chicken GIT are *L. crispatus*, *L. gallinarum*, *L. johnsonii*, *L. salivarius*, and *L. reuteri* [66]. In 1971, Morishita et al. [67] found that avian and some non-avian strains of lactobacilli (administered through drinking water) were able to establish a population and persist in the gastrointestinal tract of germfree birds. In particular, *L. plantarum* and *L. casei* of non-intestinal origin were capable of persisting in the GIT of germfree birds, although they are unlikely to be present in non-germfree birds with an in-tact GIT microflora, as they were eliminated when administered with avian isolates. *L. acidophilus* ATCC 4356,

a human pharyngeal isolate, could not be established even in the absence of other bacteria.

The crop is the first organ of the chicken GIT that is colonized [87,98] and the microflora is dominated by lactobacilli [28] (discussed further in section 1.2.3). The proventriculus, gizzard and duodenum have a relatively sparse bacterial population due to the combined effects of low pH and rapid transit time [64]. Lactobacilli were found to be a significant proportion of the intestinal microflora of broiler chickens [5]. The ileum has not been well studied when compared to the large amount of literature regarding the chicken caeca. In 1978 Salanitro et al. [73] used traditional microbiological techniques to investigate the microflora in the chicken ileum and observed predominantly facultatively anaerobic bacteria including the following: *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Escherichia coli*, *Enterococcus, Fusobacterium, Eubacterium, Clostridium* and *Propionobacterium*. In 2002 Knarreborg et al. [53] used molecular and cultural techniques to analyse the ileal microflora. Lactobacilli were found to be present at $>10^7$ CFU/g, and molecular analyses indicated that *L. johnsonii, L. crispatus, L. reuteri* and *L. salivarius* were present along with *Streptococcus* and *Clostridium perfringens*.

The caeca and colon contain a similar, large and complex bacterial population [64]. Early microscopic data suggested a thick layer of bacteria associated with the caecal tissue [28]. Because obligate anaerobes predominate in the caeca and could not be easily cultivated [64], early studies isolated mostly bifidobacteria and bacteroides [76] as well as lactobacilli [75]. The development of improved anaerobic growth conditions and media allowed for the detection of a wide range of species (reviewed in Mead, [64]). The recent development of molecular techniques has further revealed the complexity of the

caecal microflora. Zhu et al. [107] PCR-amplified the V6-V8 region of 16S rDNA from the caecal population and sequenced over 1600 individual 16S rDNA clones/TTGE (temporal temperature gradient electrophoresis) bands. Two hundred and forty-three different 16S rDNA sequences were obtained, including eubacteria and clostridia. Comparison of TTGE band sequences of the caecal contents and the caecal mucosa indicated that bacteria closely related to *L. acidophilus* were found in the mucosal samples, and cloning of 16S rDNA sequences detected the presence of *L. reuteri*, *L. plantarum*, *L. delbrueckii*, and *L. mali* in caecal mucosal scrapings. The presence of bacteria in the mucosal scrapings that are absent in the contents may suggest adherence to caecal tissue. In a similar study, *L. reuteri*, *L. oris*, *L. salivarius*, *L. acidophilus*, *L. crispatus* and *L. fermentum* 16S rDNA sequences were cloned from caecal contents [58]. Lactobacilli comprised 24% of the 16S rDNA clones, 65% of which were members of the *L. acidophilus* group. This contrasts with the results from Zhu et al. [107] who found less than 1.5% of the clones were lactobacilli. Gong et al. [34] detected a large number of butyrate reducing bacteria and only 4 *Lactobacillus* sequences of 87 total clones.

The microflora of the chicken GIT changes significantly during the early life of broilers. The crop is the first organ colonized [98], and may initially contain streptococci and *E. coli*, which are followed by lactobacilli [87] that predominate thereafter [76]. Some development of the ileal microflora has also been observed [53]. The caecal microflora can take up to 30 days to be fully established [64,76]. Culture-independent data may indicate a trend for succession of the microflora found in the GIT, starting with the crop [30,98]. Further, each bird has a unique microflora [98,107].

1.2.3. *Lactobacillus* and the chicken crop

The crop is a distensible food storage organ located midway along the chicken oesophagus that is not essential for digestion in *ad-libitum* fed birds [22]. The epithelial cells of the crop and oesophagus are stratified squamous cells. Mucous glands are present in the mouth and oesophagus. The crop itself is non-secretory and is not covered with mucous [64]. The microflora of crop is unique in that it is composed largely of lactobacilli, which form a thin layer in intimate association with the epithelium [28].

1.2.3.1. Culture dependent data

Fuller [32] observed a *Lactobacillus* population of 7.6 to 8.9 $\log_{10}$ CFU/g in the chicken crop, with a maximal population around days 4 to 6. Coliforms were present at 6.6 $\log_{10}$ CFU/g on day 2 but decreased to 4.1 $\log_{10}$ CFU/g by day 18. Streptococci were observed to peak at 6.7 $\log_{10}$ CFU/g on day 2 and stabilize at 5.2 $\log_{10}$ CFU/g by day 18. Fuller and Turvey's early work [28] investigated the population of bacteria intimately associated with the crop and other gastrointestinal tissues of the chicken. Tissues were aseptically extracted, dissected, washed and homogenized. Three washes of the tissue sections were performed before homogenization, and the anaerobic CFU counts (serial dilutions plated on MRS and other selective media) of the third wash and the homogenized tissue were compared and thee data suggested adherence (section 1.2.3.1). The crop was found to carry the highest cultivated bacterial population (and *Lactobacillus* population) as detected by colony counts on both selective and non-selective media. Inhibition of the *Lactobacillus* population in the crop encourages the growth of streptococci and enterococci [32] and strict anaerobes have not been cultivated from the crop [64]. Sarra et al. [75] isolated a large number of *L. salivarius* from the chicken crop, although *L. acidophilus* and *L. reuteri* have also been cultivated [76]. Our

research (Chapter 2 Table 2.8) confirmed this using both culture-dependent and culture-independent molecular techniques, indicating that *L. crispatus*, *L. gallinarum*, *L. johnsonii*, *L. reuteri* and *L. salivarius* are present in the chicken crop.

1.2.3.2. Effects of *Lactobacillus* crop microflora on the chicken GIT

Fuller's work indicated some potential roles of lactobacilli in the chicken crop. The production of lactic acid by lactobacilli in the crop creates an environment (pH 4.5 to 5.0 [22,64]) that can inhibit a number of non-acid-tolerant bacteria and is proposed to create a selective environment. For example, reducing the *Lactobacillus* population with antibiotics increases the crop coliform counts [29,32]). Fuller [29] speculated that adherence to the crop epithelium may allow rapid inoculation of the feed, thereby conferring an advantage to the lactobacilli and reducing the ability of potential pathogens or undesirable bacteria to establish in the crop and the remainder of the GIT. It is controversial whether the crop and its microflora participate in digestion, however lactobacilli isolated from the crop have been shown to produce amylases [18]. It has been proposed that amylase activity of crop isolates may be beneficial for both the bacteria and the host [18]. Fuller and Brooker [30] noted that the sloughing of crop epithelial cells could carry the adherent lactobacilli and inoculate other areas of the GIT indicating the potential importance of the crop microflora.

1.2.3.3. Adherence of lactobacilli to the crop epithelium

By comparing the CFU/g obtained from crop tissue homogenate and crop tissue washes, Fuller and Turvey [28] postulated that the presence of adherent bacteria would be indicated by higher counts in the tissue homogenate. The crop showed a 10-fold difference between the CFU/g in the homogenate and third wash, thereby suggesting the

crop carried adherent bacteria. The same pattern was observed in other sections of the GIT but the difference was not as great as that seen for the crop. Fuller and Turvey [28] also investigated bacterial association with the crop, ileum, and caecum using histology and Gram staining. The crop was observed to contain a layer of Gram-positive bacilli up to two or three cells thick, forming a nearly complete layer. Scrapings of crop tissue also revealed Gram-positive bacilli adhered to individual epithelial cells, although birds that had feed withheld for 12 hours did not possess bacteria in the crop. Sloughing of the crop epithelium (with attached bacteria) was postulated to explain these results. A different pattern of adherence was observed in the ileum and caeca, where few adherent Gram-negative bacteria per cell and thick layers of Gram-positive bacilli were observed, respectively. Fuller and Turvey [28] concluded that the crop contains a large *Lactobacillus* population that appears to be tightly and uniformly adherent to the crop epithelium, although the histological data suggested the caeca contained more bacteria. Fuller drew an analogy between the adherence of lactobacilli in the crop to that observed in the rat stomach, which has been well studied (for example, [89,91]).

Fuller [29] also studied the adherence of avian and non-avian isolates to *ex vivo* chicken crop tissue. For adherence assays, fasted chickens that lacked adherent *Lactobacillus* were euthanised and the crop was aseptically removed. The epithelium was scraped with a glass slide to free epithelial cells, which were exposed to *Lactobacillus* cultures. Microscopic examination revealed that only avian lactobacilli were capable of adhering to *ex vivo* chicken crop cells (as observed in [67]); bacterial isolates from other animals did not adhere. Fuller [29] also noted that some adherent lactobacilli appeared to be related to *L. salivarius* and *L. fermentum* (as determined by culture-dependent

biochemical tests), yet strains of these species from culture collections did not adhere. This is not surprising given that culture collection strains may not be of avian origin, Further, adherence of avian strains can also be lost after repeated subculturing [79]. Investigation of the *ex vivo* crop tissue from chickens of different ages revealed that some *in vitro* cultured lactobacilli adhered to the tissue obtained from chickens of all ages [29]. It seems plausible that the avian isolates tested carried and expressed genes necessary for adherence that are absent or silent in non-adhering isolates from other animals. These genes may become silent after repeated subculturing. Fuller [29] also sampled chicken feed and egg shells and the air from brooder rooms for the presence of lactobacilli. *Lactobacillus* isolates found in one broiler feed mix, the brooder house air and the eggshells were capable of adhering to *ex vivo* chicken crop cells. It is interesting to consider the origin and subsequent ability of these environmental isolates to bind chicken crop cells from the molecular perspective: did they carry/express genes to promote adherence which would be absent or inactive in isolates from other sources?

Work by Fuller and Brooker in 1974 [30] investigated the interactions of lactobacilli and *ex vivo* chicken crop tissue in greater detail using both light microscopy and transmission electron microscopy (TEM). They proposed that carbohydrate residues on both bacteria and crop cells are responsible for adherence to the crop epithelium based on their *in vitro* inhibition tests and microscopy data. A 7 nm gap was found to exist between adhering Gram-positive bacteria and the chicken crop and was usually filled with carbohydrate rich material, and "bridges" were also observed linking bacteria together. Filaments were also observed in the *Lactobacillus*-crop epithelial cell gap, but

where thought not to be essential as stationary phase cultures lacking such filaments were also able to adhere.

In 1975, Brooker and Fuller [15] studied the carbohydrate nature of the adherence determinants in greater detail. They studied two strains of lactobacilli *in vitro*: strain 59, a chicken isolate belonging to biotype B (likely related to *L. salivarius* or *L. aviarius* [29,94]) known to adhere to chicken crop cells and *L. acidophilus* NCTC 1723, a rat isolate shown not to adhere to crop epithelial cells [15]. The adherence of strain 59 to *ex vivo* crop cells was also determined. Different staining techniques were used to detect carbohydrates. Ruthenium red staining of strain 59 *in vivo* detected three distinct layers of carbohydrate rich material; two of which are located in the cell wall and are observed with non-specific staining and a third, diffuse, extracellular material containing a number of filaments. This diffuse material was observed in the 7 nm gap between the lactobacilli and *ex vivo* crop epithelial cells. Strain 59 grown *in vitro* contained this diffuse material, although the filaments were observed to connecting cells together and were not present on the side of the cell not adjacent to other cells. *L. acidophilus* NCTC 1723 had a very thin outer layer and when grown *in vitro*. Colloidal iron staining revealed thin layers of carbohydrate on strain 59 and crop cells but did not stain *L. acidophilus* NCTC 1723. Alcian Blue-Lanthanum Nitrate (ABLN) also detected the extracellular floccular carbohydrate material on strain 59 grown *in vitro* and *in vivo*, and did not detect a similar layer for *L. acidophilus* NCTC 1723. All the results suggest that there is a carbohydrate rich layer on the surface of the adherent strain 59.

Bayer et al. [7] also studied the ultrastructure of the chicken crop with scanning electron microscopy. They found bacteria adhering to both crop tissue and feed particles

and observed epithelial cell sloughing in the areas furthest away from the oesophageal entry and exit [7]. In 1983, Watkins and Miller [105] studied the ability of an *L. acidophilus* isolate (from a feed probiotic, Acidolac™, source of the strain was unspecified) to colonize the GIT of germfree birds and examined tissue sections for the presence of adherence. They found that the *L. acidophilus* isolate adhered to crop epithelial cells, however appeared to do so intermittently, unlike the observations made by Fuller [28]. Interestingly, their TEMs of crop tissue show bacilli adhering tightly to the crop cells and also adhering to the crop epithelium with a gap [105], as Brooker and Fuller observed [15]. This could suggest that several different methods of adherence mediate the interaction between the crop *Lactobacillus* population and the crop tissue.

The identity of the carbohydrate factors linking lactobacilli and chicken crop epithelium was not determined, as treatment with various carbohydrate cleaving enzymes did not affect adherence [30]. Concavalin A, a protein known to bind carbohydrates [103], was successfully used to inhibit adherence of lactobacilli to *ex vivo* chicken crop epithelial cells [30]. In a subsequent paper, [31] Fuller suggested that adherence of strain 59 to *ex vivo* crop cells did not change with altered temperature, pH, or nutrient conditions studied. However, the reduction in adherence upon periodate treatments of the isolate (due to degradation of carbohydrate moieties) agreed with the previous suggestion that carbohydrates are an important adherence determinant. Of note, however, were the observations that 1) treatment of the cells with proteinases reduced adherence and 2) treatment with proteinase caused release of carbohydrates into the culture medium. It is therefore possible that the adherence determinants that Fuller studied were glycoproteins on the bacterial cell surface.

The data from Fuller's research, generated by traditional cultural and biochemical tests and *in vitro* adherence assays, were very important in understanding the potential roles of lactobacilli as members of the chicken gastrointestinal microflora. Fuller clearly demonstrated the intimate and specific association of lactobacilli with the chicken crop and made significant progress in elucidating potential adherence mechanisms. However, it is important to understand the confines inherent in Fuller's research in the light of current investigative tools. Foremost is the method by which he classified the *Lactobacillus* isolates (i.e. biochemical tests), a method that has since been replaced by more rapid, reproducible, and detailed molecular analyses [77]. In addition, techniques such as DGGE (denaturing gradient gel electrophoresis) are now available to study the species-specific changes in the gastrointestinal microflora with the development of the bird (Chapter 2). Molecular techniques for investigating the interactions between bacteria and host such as *in vivo* expression technology [104] and tracking specific strains by antibiotic resistance plasmids or marking bacteria with fluorescent labelling [33] allows greater investigation into the ability of single strains to persist and survive in the GIT. Therefore, the conclusions that can be drawn from Fuller's research regarding adherence of specific isolates via carbohydrate residues must be considered within the context of his experiments and new data. In our analysis of the ecology of the chicken crop (Chapter 2), we observed a number of *L. acidophilus* group species to be present. We therefore began our investigation of their ability to persist and potentially adhere to crop tissue by looking at cell surface factors on these isolates.

1.3 Key genetic and biochemical characteristics of gastrointestinal *L. acidophilus*

1.3.1: Overview

The development of the gastrointestinal microflora is a complex interaction between the host and resident bacteria and also among diverse communities of bacteria within the gastrointestinal tract. For example, many host factors such as the pH, GIT motility, physiology, the presence of mucous and the rate of exfoliation all affect the ability of bacteria to persist and thrive in the GIT environment [38]. The diet may also influence the bacterial populations that can survive in the GIT. Several bacterial factors are also important for survival and growth in the GIT. Bacterial metabolism is important, as the bacterial energy and substrate requirements must be met by the host, the diet, or other bacteria. Therefore, the ability of lactobacilli to ferment organic materials under anaerobic conditions likely assists them in gastrointestinal survival. Further, cell surface associated proteinases [57] likely aid the lactobacilli in obtaining nutrients for survival. In general, with Gram-positive bacteria surface factors such as a thick peptidoglycan cell wall [70], spores or capsules may also be important due to their protective role [38]. Indeed, lactobacilli may express extracellular polysaccharide (EPS, although its potential presence and function in the GIT is uncertain) [45], and have been shown to produce proteinaceous coats known as S-layers (see below). For organisms that survive in the lower gastrointestinal tract, resistance to bile salts is also important for survival [51], therefore the expression of enzymes such as Bile Salt Hydrolase (BSH) [20] may be advantageous. Lactobacilli have been shown to carry *bsh* genes [54]. Antimicrobial compounds also affect survival (for review, see [71]), and if the host animal is regularly ingests antibiotics (as seen with farm animals including the chicken), then resistance to

those antibiotics will also be an essential survival characteristic of gastrointestinal bacteria [71]. Lactobacilli are known to be resistant to a number of antibiotics [42]. One of the most important properties of gastrointestinal bacteria is acid tolerance [51]. Bacteria encounter a low pH environment as they pass through the stomach due to host acid secretion, and also in specialized organs such as the chicken crop, which are populated by lactobacilli that lower the pH as well [22]. Although little information has been published regarding potential mechanisms of acid tolerance of lactobacilli, their ability to resist acid is a well-established fact [19].

In addition to conferring survival in the GIT, these characteristics also appear to provide a mechanism whereby lactobacilli and other LAB survive and grow in foods. Indeed, given that LAB are part of the microflora of many foods and can ferment plant and animal organic materials that contain sufficient carbohydrate content, it is not surprising that they are also members of the gastrointestinal microflora. The presence of LAB in such environments undoubtedly contributed to the natural fermentation of food in ancient times, and have been utilized by humans for preservative purposes for hundreds, if not thousands of years [94]. There is archaeological evidence for ancient (i.e. older than 3000 years before common era [BCE]) fermented food production from Switzerland, Egypt, and Mesopotamia, including references to cheese and butter/yoghurt in cuneiform tablets for Uruk. However, modern fermentation technologies using LAB rely on information derived from molecular biology, including species and strain designations of starter cultures, selection of optimized starter organisms based on characteristics such as bacteriophage resistance, rapid growth and acid production, and tolerance to low pH [96], and genetic engineering for starter and non-starter organisms (for review, see de Vos

[21]). A considerable amount of time and money is now invested in optimization of food fermentation processes, with a heavy reliance on molecular biology.

According to Vaughan et al. [99], colonization is crucial for survival and persistence of bacteria in the GIT. Colonization can occur via a variety of methods and may involve aggregation or biofilm development. The following general factors have been proposed to be involved in colonization and persistence of the *L. acidophilus* group: aggregation (*L. gasseri*, *L. johnsonii*, *L. crispatus* and *L. acidophilus* have been shown to autoaggregate [14,17,25,55,101]); protective surface factors; and adherence to tissue components, gastrointestinal mucous and/or cell lines, as demonstrated *in vitro* [1,3,17,36,48,50,51,60,62,69,79-81,95]. Specific cellular components involved in thee processes include Lipoteichoic acid (LTA, sugar phosphate polymers inserted in the cell membrane via a lipid moiety [70]) and S-layers (macromolecular aggregates of S-proteins outside the cell wall [74]). LTA was found to be responsible for adherence of *L. johnsonii* to Caco-2 cells [35] and *L. acidophilus* isolates to the mouse GIT [80] and pig stomach [92].

S-layers have been shown to be responsible for adherence to cell lines and tissue components *in vitro* (discussed below) and may also be responsible for aggregation [55]. Schneitz et al. [79] observed an *L. acidophilus* chicken isolate with an S-layer. This strain adhered to *ex vivo* chicken intestinal epithelial cells, although repeated subculturing on MRS resulted in no adherence as observed by light microscopy, TEM analysis suggested that the S-layer on the non-adherent *Lactobacillus* was covered with another layer of diffuse material, and this phenotype was associated with different colony morphology, lack of aggregation in broth culture, and failure to protect against *Salmonella* challenge in

chickens. The outermost layer in the subcultured variant could be EPS (as observed by [60]), although the authors did not investigate its properties further. Kos et al. [55] analysed the ability of *L. acidophilus* M92 to adhere to porcine ileal sections and found that extraction of the S-layer with LiCl reduced the adherence as observed by light microscopy. Autoaggregation of *L. acidophilus* M92 in broth culture was also reduced upon removal of the S-layer, suggesting that either aggregation proteins were extracted when the S-layer was removed or that the S-layer is involved in aggregation.

Toba et al. [95] studied the ability of *L. crispatus* JCM 5810 (isolated from chicken faeces) to adhere to specific tissue components. This strain is characterized by strong adherence to insoluble type-I and -IV collagens and laminin (obtained from human and mouse tissues). Adherence to collagen-IV was inhibited by collagen-I and -IV. Adherence to Matrigel, a commercial basement membrane preparation containing collagen-IV and laminin, and the ECM (extracellular matrix) extracted from Intestine 407 cells was also observed. Horie et al. [39] observed dose-dependent inhibition of enterotoxigenic *Escherichia coli* (ETEC) to Matrigel by *L. crispatus* JCM 5810, likely via competition for laminin binding sites. The ability to adhere to collagens and laminin, and inhibit ETEC was found to be associated with the S-protein [39,95] (refer to section 1.3.2.2 for further information).

Based on the fact that S-layers form the outermost surface of the bacterial cells in the absence of EPS, and are composed of many S-protein subunits [82] the S-proteins of chicken lactobacilli (Chapter 2) were initially characterized as potential GIT persistence factors (Chapter 3).

1.3.2: S-layers of the group A *acidophilus*

1.3.2.1: Overview

Masuda [63] first observed that only members of *L. acidophilus* group A species contain S-layers, those species from group B do not. S-layers are proteinaceous, two-dimensional structures associated with the peptidoglycan surface of bacteria and archaea [74]. S-layers form the outermost layer of the cell in Gram-positive bacteria lacking a capsule and therefore are expected to play a role in mediating the interactions between the bacterium and its environment [82]. These layers have been shown to recognize and adhere to surfaces (as discussed in 1.3.1, above) as well as acting as a [protective] barrier that allows selective transport of nutrients into the bacterial cell [82]. S-layers are generally composed of a single (glyco)protein species (the S-protein) ranging in size from 40-200 kDa molecular weight [74]. These secreted proteins form crystalline lattices with hexagonal, square, or oblique symmetry on the surface of the bacterial cell [82].

Glycoprotein S-layer subunits contain carbohydrate structures of 20 to 50 repeating units covalently bound to the protein moiety [74]. S-layers contain regular, identical pores that cover a total of 30-70% of the surface area of the layer [82]. Non-covalent interactions (including hydrogen bonding) mediate subunit interactions [74]. The vast majority of S-proteins sequenced to date have an N-terminal signal peptide that directs secretion of the S-protein. The primary amino acid sequence of most mature S-proteins contain few sulphur-containing amino acids and have a bias toward glutamine and asparagine [82]. *Lactobacillus* S-proteins differ significantly from their counterparts in other bacteria (Table 1.1). In particular, the S-proteins of lactobacilli do not contain S-layer homologous (SLH) motifs, *ca.* 50 amino acid repeats found in the N-terminus of many S-layers and the C-terminus of cell wall associated enzymes in Gram-

Table 1.1. Differences between S-layers in lactobacilli and other bacteria

Characteristics of *Lactobacillus* S-proteins	**General characteristics of S-proteins from other bacteria**
Glycosylation rare[1] [36]	May be glycosylated
N-terminal variability, C-terminal conservation [11,84]	N-terminal conservation, C-terminal variability
C-terminus involved in cell wall anchoring [11,84]	N-terminus involved in cell wall anchoring
No SLH motifs[2] [74]	SLH motifs common
High pI (≥9.5) [13]	Low pI
Small in size (40-50 kDa) [74]	Large in size (up to 200 kDa)

[1] To date only two *Lactobacillus* strains have been found to express glycosylated S-proteins: *L. buchneri* 41021/251 and *L. plantarum* 41021/252 [68].
[2] *L. acidophilus* has two repeats in C-terminal domain that are not homologous to SLHs.

positive bacteria [74]. The structures produced by *Lactobacillus* S-layers analysed to date form oblique symmetry. More information regarding specific S-proteins observed in lactobacilli can be found in Tables 1.2 (the GAA) and 1.3 (others).

1.3.2.2. Molecular analysis of S-protein genes

After the discovery of S-proteins in the Group A *acidophilus*, Masuda [63] analysed them by digesting extracted S-proteins with proteases (peptide mapping) and found that primary structural differences existed between different strains of the same species. Among the GAA, the S-proteins of the *L. acidophilus* and *L. crispatus* species have been characterized to date (Table 1.2). The first and most characterized S-proteins of the GAA are SlpA and SlpB (initially referred to as S_A and S_B, [9]) of *L. acidophilus* ATCC 4356. In 1993, Boot et al. [8] undertook the analysis of SlpA protein *L. acidophilus* ATCC 4356. Since no sequence information was available for *Lactobacillus* S-protein genes, they extracted S-protein from a liquid culture with guanidine hydrochloride and purified it with a cation exchange column. Antibodies to the S-protein were raised in BALB/c mice, which were used to screen a phage library containing genomic DNA from *L. acidophilus* ATCC 4356. Selected transformed phage were then subjected to Southern analysis using an oligonucleotide probe designed from a peptide fragment sequenced from SlpA, and a 4 kb fragment was cloned into pUC19 and pBluescript. The S-protein gene (*slp*A) was subsequently expressed in *E. coli* transformed with these recombinant plasmids, as detected by Western analysis using the S-protein antibody. Sequence analysis of the cloned fragment revealed the presence of a 1.332 kb open reading frame (ORF) (Fig. 1.3A) encoding a protein of 444 amino acids with a predicted molecular weight of 44.3 kDa. The 5' end of the gene was initially

Table 1.2. S-proteins characterized from group A *Lactobacillus acidophilus* spp.

Gene name (gene size [kb], protein size [aa])	Organism (GenBank Accession Number)	Regulation	Other properties	Reference
Gene has been characterized				
*slp*A (1.332, 444)	*L. acidophilus* ATCC 4356 (X89375)	Expressed in laboratory culture. Has two promoters (P2 starting at –335 nt and P1 starting at –228 nt) although only P1 is active *in vitro*. Rho-independent terminator observed downstream. mRNA has a 15 min half-life.	Confirmed non-glycosylated. Crystallisation associated with N-terminus.	[8-10,12,84-86]
*slp*B (1.368, 456)	*L. acidophilus* ATCC 4356 (X89376)	Silent in laboratory culture	ND	[9]
*cbs*A (1.317, 440)	*L. crispatus* JCM 5810 (AF001313)	Expressed in laboratory culture	Binds collagen-I and -IV and laminin. Binds *ex vivo* chicken colon section. Adheres to Matrigel and ECM extract from Intestine 407 cell line. Crystallisation and adherence associated with N-terminus.	[1,62,81,95]
*cbs*B (1.356, 452)	*L. crispatus* JCM 5810 (AF079365)	Silent in laboratory culture	Does not bind collagen-I and -IV and laminin	[81]
*slpn*A (1.374, 458)	*L. crispatus* LMG 12003 (AF253043)	ND	Does not bind collagen-I and -IV and laminin (as per [81])	ND
*slpn*B (1.317, 439)	*L. crispatus* LMG 12003 (AF253044)	ND (Silent in laboratory culture as per [81])	Does not bind collagen-I and -IV and laminin (as per [81])	ND

[1]ND – Not done, i.e. not studied in literature/not published

Table 1.2. Continued

Gene name (gene size [kb], protein size [aa])	Organism (GenBank Accession Number)	Regulation	Other properties	Reference
Gene has been characterized				
*lbs*A (1.398, 466)	*L. crispatus* MH315 (AB110090)	ND	ND	ND
*lbs*B (1.326, 442)	*L. crispatus* MH315 (AB110091)	ND	ND	ND
s-layer (1.353, 451)	*L. crispatus* M247 (AJ007839)	ND	ND	ND
Gene has not been characterized; putative S-layer based on microscopy and/or SDS-PAGE				
None given (ND[1])	*L. acidophilus* JCM 1034 (ND)	ND	Hemagglutinates sheep erythrocytes	[106]
None given (ND)	*L. acidophilus* (ND)	ND	Binds *ex vivo* chicken intestinal epithelia	[79]
None given (ND)	*L. acidophilus* M92 (ND)	ND	Responsible for aggregation, adheres to porcine ileal tissue	[55]
None given (ND)	*L. acidophilus* CRL 639 (ND)	ND	Does not appear to be involved in collagen-I and fibronectin binding	[60]

[1]ND – Not done, i.e. not studied in literature/not published

Table 1.3. Summary of S-proteins characterized from lactobacilli other than the *L. acidophilus* group.

Gene name (gene size [kb], protein size [aa])	Organism (GenBank Accession number)	Regulation	Other properties	Reference
Gene has been characterized				
*slp*A (1.335, 484)	*L. brevis* ATCC 8287 (Z14250)	Expressed in Laboratory culture. Rho independent terminator and 2 upstream promoters detected (-163 and –77 nt). Both promoters active but closer promoter is predominant. mRNA has 14 min half-life.	Adheres to Intestine 407, bladder T24 and EA-hy926 and Caco-2 human cell lines. Adheres to fibronectin. Adherence is associated with N-terminus.	[46,102]
*slp*B (1.449, 483)	*L. brevis* ATCC 14869 (AY040846)	Expressed in laboratory culture (aerobic and anaerobic)	ND	[43]
*slp*C (1.383, 461)	*L. brevis* ATCC 14869 (AY040847)	Silent in laboratory culture	ND	[43]
*slp*D (1.239, 413)	*L. brevis* ATCC 14869 (AY040848)	Expressed in laboratory culture (aerobic only)	ND	[43]
s-layer (1.317, 439)	*L. helveticus* IMPC HLMI (AJ388564)	ND	ND	[100]
s-layer (1.320, 440)	*L. helveticus* IMPC M696 (AJ388563)	ND	ND	[100]
s-layer (1.320, 440)	*L. helveticus* IMPC i60 (AJ388562)	ND	ND	[100]
s-layer (1.314, 438)	*L. helveticus* CNRZ 35 (AJ388561)	ND	ND	[100]

[1]ND – Not done, i.e. not studied in literature/not published

Table 1.3. Continued

Gene name (gene size [kb], protein size [aa])	Organism (GenBank Accession number)	Regulation	Other properties	Reference
s-layer (1.320, 440)	*L. helveticus* CNRZ 303 (AJ388560)	ND	ND	[100]
s-layer (1.320, 440)	*L. helveticus* ATCC 15009 (AJ388559)	ND	ND	[100]
s-layer (1.320, 440)	*L. helveticus* ATCC 12046 (AJ388558)	ND	ND	[61,100]
*slp*H1 (1.320, 440)	*L. helveticus* CNRZ 892 (X91199)	ND	ND	[16]
*slp*H2 (1.320, 440)	*L. helveticus* CNRZ 1269 (X92752)	ND	ND	ND
s-layer (1.170, 390)	*L. helveticus* JCM 1008 (AB061778)	ND	ND	ND
s-layer (1.191, 397)	*L. helveticus* JCM 1007 (AB061777)	ND	ND	ND
s-layer (0.267, 89, [partial])	*L. helveticus* (AF247817)	ND	ND	ND
Gene has not been characterized; putative S-layer based on microscopy and/or SDS-PAGE				
None given (ND[1])	*L. buchneri* 41021/251 (ND)	ND	Glycosylated	[68]
None given (ND)	*L. plantarum* 41021/252 (ND)	ND	Glycosylated	[68]

[1]ND – Not done, i.e. not studied in literature/not published

A.

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L. acidophilus ATCC 4356 slpA  ATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGCTGCTTTACTTGC  50
L. acidophilus ATCC 4356 slpB  ATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGC---TTTACTTGC  47
L. crispatus JCM 5810 cbsA     ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
L. crispatus MH315 lbsB        ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
L. crispatus LMG 12003 slpnB   ATGAAGAGAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
L. crispatus M247 s-layer      ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
L. crispatus MH315 lbsA        ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
L. crispatus JCM 5810 cbsB     ----------------------GTGAGCGCTCCTGCTGCTGCTTTATTAGC  29
L. crispatus LMG 12003 slpnA   ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC  50
                                                    ** ****** *******   **** * **

L. acidophilus ATCC 4356 slpA  TGTTGCTCCAGTTGCTGCTTCTGCTGTATCTACT------GTTAGCGCTG  94
L. acidophilus ATCC 4356 slpB  TGTTGCTCCAGTTGCTGCTTCTGCTGTATCTACT------GTTAACGCTG  91
L. crispatus JCM 5810 cbsA     TGTTGCTCCAGTTGCTGCTTCAGCTGTTTCT---------GTTAACGCTG  91
L. crispatus MH315 lbsB        TGTTGCTCCTGTCGCTGCTTCTGCTGTTTCT---------GTTAACGCTG  91
L. crispatus LMG 12003 slpnB   TGTTGCTCCTGTCGCTGCCTCAGCTGTTTCT---------GTTAACGCTG  91
L. crispatus M247 s-layer      TGTTGCTCCTGTCGCTGCTTCAGCCGTTTCT---------GTTAACGCTG  91
L. crispatus MH315 lbsA        TGTTGCTCCTGTCGCTGCTTCTGCTGTATCTACTGTTTCAGCTGATGCTG 100
L. crispatus JCM 5810 cbsB     TGTTGCTCCTGTCGCTGCTTCTGCTGTTTCT---------GTTAACGCTG  70
L. crispatus LMG 12003 slpnA   TGTTGCTCCTGTCGCTGCCTCAGCTGTTTCT---------GTTAACGCTG  91
                               ********* ** ***** ** ** ** ***          * *   ****

L. acidophilus ATCC 4356 slpA  ---------CTACTAC---------TATTAACGCAAGTTC----ATCAGC 122
L. acidophilus ATCC 4356 slpB  ---------CCGCTG------------TTAATGCTATTGCT---GTTGGC 117
L. crispatus JCM 5810 cbsA     ACGCCGTTTCAAGTGCAAACAACAGTAATTTAGGTAATAATAACAATGGT 141
L. crispatus MH315 lbsB        ---CAAGCTCAAGTGC---------TGTTCAAACTGCTACCAACATTGGT 129
L. crispatus LMG 12003 slpnB   ------------ACGC---------CGTTCAATCAGCTACTCAACTTGGT 120
L. crispatus M247 s-layer      CTGACAACACCGTTGCAACCACTACCAACACAGCAAACACTGTAATTAAT 141
L. crispatus MH315 lbsA        CTGCAACTACTACTGCAACTACTAACAGCAATGTTACTCTTAACTTAAAC 150
L. crispatus JCM 5810 cbsB     CT---GACTCTACTGCAACTACTACTGCTAAAGCTACTGATTATACCAAC 117
L. crispatus LMG 12003 slpnA   CT---GACTCTACTGCAACTACTACTGCTAACGCTACTGACTACACTAAT 138

L. acidophilus ATCC 4356 slpA  AATCA-----ATACCAACACTAA-TGCTAAGTACGATGTTGATGTAACTC 166
L. acidophilus ATCC 4356 slpB  GGTT-------CAGCTACCCCAT-TACCAA--ACAACTCAGATGTACAAA 157
L. crispatus JCM 5810 cbsA     ACTTTC-ACTGTTTTACCATTAAATAACGGTGCTACTGTTAATGTTAAGC 190
L. crispatus MH315 lbsB        ACT-------GTTTTACCATTAACTGATGGTTCTACTGTTAACGTTAAGC 172
L. crispatus LMG 12003 slpnB   ACT-------GTACCTGCTTTATCAAACGGTGACACTGTTAACGTTAAGC 163
L. crispatus M247 s-layer      GCTG---ATGGTACCGCAATCAACACTCCA-GCTGATGCAAAATACGATG 187
L. crispatus MH315 lbsA        GGTGCAGGTAGTACTGCAACCGA-TGCTGCTAACACTGTTAATGTATCAT 199
L. crispatus JCM 5810 cbsB     ATCA------ATCTTGGTGGCTC-AGCTGTTTCAAACAATGAAAACCAAG 160
L. crispatus LMG 12003 slpnA   ATTA------ATTTAGGCGGTAC-AACTGTTTCAAATACTGAAAATCAAG 181

L. acidophilus ATCC 4356 slpA  CTAGTGTTTCTGCAGTTGCTGCAAATACTGCTAACAACACTCCAGCTATT 216
L. acidophilus ATCC 4356 slpB  TTAGTTCATCAGTTGCTGGTGTAACTACTAAGAATGGCTCAAGCTACACT 207
L. crispatus JCM 5810 cbsA     CAAACATTTCATTGAACACTTCAGCATACGAAGGTGTTAAGGCAAACATT 240
L. crispatus MH315 lbsB        CAAACATTTCATTGAACACTTCAGCATACGAAGGTGTTAAGGCAAACATT 222
L. crispatus LMG 12003 slpnB   CAAATGTTTCATTAAACACTTCAGCTTATGAAGGTGTTAAGGCAAACATC 213
L. crispatus M247 s-layer      TTGATGTAACACCTAACCTTACTGCTACTGCAGCTTCTACTGTAAATGGA 237
L. crispatus MH315 lbsA        CAAACTTTAGCTTAAACGCACCAGTTAAGGCTAATAACGCTGTAACTGCT 249
L. crispatus JCM 5810 cbsB     TAGACGTAACTCCAGCTCTTACTCTTAATGGAA----CAAAG-------- 198
L. crispatus LMG 12003 slpnA   TTGACGTAACTCCAAGCATTGTTCTTAATGGTAATGTCAAGAATACTGCT 231
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Fig. 1.3. Comparison of group A *acidophilus* S-protein genes. **A**. ClustalW alignment. "*" indicates identical nucleotide in all sequences. Box indicates DNA encoding the signal sequence. Arrow indicates start of DNA encoding the C-terminal anchor in *slp*A. The GenBank accession numbers are as follows: ATCC 4356 *slp*A, X89375; ATCC 4356 *slp*B, X89376; JCM 5810 *cbs*A, AF001313; JCM5810 *cbs*B, AF079365; LMG 12003 *slpn*A, AF253043; LMG 12003 *slpn*B, AF253044; M247 *s-layer*, LCR7839; MH315 *lbs*A, AB110090; MH315 *lbs*B, AB110091. **B**. Phylogenetic tree based on DNA sequence comparisons. **C**. Percent identity between DNA sequences.

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L. acidophilus ATCC 4356 slpA  GCCGGTAACCTTACTGGTACTATTTCAGCAAGTTACAATGGTAAGACTTA 266
L. acidophilus ATCC 4356 slpB  AACGGTAGAATTTCTGGTTCTATCAACGCTTCTTACAACGGTACAAGCTA 257
L. crispatus JCM 5810 cbsA     TCAG-TATC-----------ATTCTCAGCAACTGTTGACGGTACTACTGC 278
L. crispatus MH315 lbsB        TCAG-TATC-----------ATTCTCAGCAACTGTTGACGGTACTACTGC 260
L. crispatus LMG 12003 slpnB   TCAG-TATC-----------ATTCTCAGCTACTGTTAATGGTACTACTGC 251
L. crispatus M247 s-layer      CAAACTATT-----------AACGGTAGC-ATTACTGGTAACATTACTGC 275
L. crispatus MH315 lbsA        GATGCTACTCTTGGTGGTGAATTAACTGCTACTCTTAACGGTACTAGTGT 299
L. crispatus JCM 5810 cbsB     GGTAACATTA----------------AGGCTAGCTTAACTGGCTCAATCAC 233
L. crispatus LMG 12003 slpnA   GGTAACCTTAT---------TTCAAAGGCTACTTTGAGTGGTTCTATTTC 272
                                                    **          *

L. acidophilus ATCC 4356 slpA  TACTGCTAACTTAAAGGCAGATACTGAAAATGCCACTATTACTGCTGCTG 316
L. acidophilus ATCC 4356 slpB  TTCAGCAAACTTTAGTTCATCAAATGCAGGTGTTGTTGTTTCAACTCCAG 307
L. crispatus JCM 5810 cbsA     TACCTCTAACTTCACTCCAAATGCTTCAACTATTGAACTTTGGAAGAATG 328
L. crispatus MH315 lbsB        TACCTCTAACTTCACTCCAAATGCTTCAACTATTGAACTTTGGAAGAATG 310
L. crispatus LMG 12003 slpnB   TGTTTCAAACTTTAAGCCAGGTGCTTCAGAAATTTCACTTTGGAAAGTTG 301
L. crispatus M247 s-layer      TAGTTACAATGGCCAATCATACACT---GGTACTTTAGATACTAAGAATG 322
L. crispatus MH315 lbsA        ATCATCAAGCTTAGCTGACGCTGCTCAAGACGTGACTGTTTCTGATGGTA 349
L. crispatus JCM 5810 cbsB     TGCATC--ATTTGGTGGTAAGAGCTTT-ACTGCTAACTTAACTGGTACTG 280
L. crispatus LMG 12003 slpnA   AGCAAC--TTTCGGTGGCAAGAGCTAC-ACTGCTAACTTAAGAGGTACTG 319
                                                  *

L. acidophilus ATCC 4356 slpA  GTAGCACTA---CTGCCGTTAAAC---CTGCTGAATTAGCTGCAGGTGTG 360
L. acidophilus ATCC 4356 slpB  GCCATACTGAACTTAGTGGTGAACAAATTAACGGTCTTGAACCAGGTAGT 357
L. crispatus JCM 5810 cbsA     AAAAGGA----TAAGGTTAC--CCAAGTAACTGAT-TTACAACAAGTAAC 371
L. crispatus MH315 lbsB        AAAAGGA----TAAGGTTAC--CCAAGTAACTGAT-TTACAACAAGTAAC 353
L. crispatus LMG 12003 slpnB   AAAAGGA----CAAGGTTAC--TCAAGTAACTGAT-TTACAAAAAGTAAC 344
L. crispatus M247 s-layer      GTAAAGT----TTCTGTAGC--TGACTCAAAGGGCACTGCTGTTACTGAT 366
L. crispatus MH315 lbsA        AGACTAACCTTTATAGCTACAACAAGGAAACTAAGAAAGTTGAAAATAAC 399
L. crispatus JCM 5810 cbsB     AACAAAA----CAACGTTACAATCAA--TGGCAATGCTGCTAAGGATGAA 324
L. crispatus LMG 12003 slpnA   ACCAAAA----CAACGTTTTAATTAA--CGGTAGAACTGCTAAAGATGAA 363
                                                          *

L. acidophilus ATCC 4356 slpA  GCTTACACTGTAACTGTTA---ACGATGTTTCATTTAACTTCGGTTCAGA 407
L. acidophilus ATCC 4356 slpB  GCTGTAACTGTTACTTTAAGAGATGGTGTTTCATTTAACTTTGGTTCAAC 407
L. crispatus JCM 5810 cbsA     TTCATCAAAC--GCTGGT------GCT---ACTTACCAAGTTAAGATGAC 410
L. crispatus MH315 lbsB        TTCATCAAAC--GCTGGT------GCT---ACTTACCAAGTTAAGATGAC 392
L. crispatus LMG 12003 slpnB   TTCATCAAAC--GCTGGT------GCT---ACTTACCAAGTTAAAATGAC 383
L. crispatus M247 s-layer      TTCTCAAAACTTACTAAT------GGT---TCATACACTGTTACTGTAAG 407
L. crispatus MH315 lbsA        TTGAACAACGTTGTTGCT------GGTCAATCATACACTCTTACTCTTAC 443
L. crispatus JCM 5810 cbsB     TTGGCTAATGTTAACGCT------GGCGACACTGTAACTGTTAGTGTAGC 368
L. crispatus LMG 12003 slpnA   TTAAGTAACGTTAATGCT------GGCAGCTCCAACACTATTACTATCAA 407
                                  *                 *     *      *

L. acidophilus ATCC 4356 slpA  AAATGCAGGTAAGACTGTTACCCTTGG---TTCAGCTAAC---TCAAATG 451
L. acidophilus ATCC 4356 slpB  TAATGCTAACAAGACTATTACTTTAGCATTTCCAAAGAACGTATCAGCTG 457
L. crispatus JCM 5810 cbsA     TCAAGTTGGCTTGAACTTCGGTTCACAAAACGCTAACAAGAAGGTTACTT 460
L. crispatus MH315 lbsB        TCAAGTTGGCTTGAACTTCGGTTCACAAAACGCTAACAAGAAGGTTACTT 442
L. crispatus LMG 12003 slpnB   TAATGTTGGTTTGAACTTTGGTTCACAAAATGCTAACAAGAAGATTACTT 433
L. crispatus M247 s-layer      TGGCGTATCATTCAACTTTGGTACTGCTAACGCAAACAAGACTATCACTC 457
L. crispatus MH315 lbsA        TAACGTTGGCTTCAGCTTTGGCTCAGCAATGAAGAACAAGACTGTTACTG 493
L. crispatus JCM 5810 cbsB     AAACGTTGGCTTTAACTTTGGTTCAGAAAACAAGGGTAAGAAAGTAACCT 418
L. crispatus LMG 12003 slpnA   AAATGTTGGATTTAACTTTGGCCCAGAAAACAAGGGTAAGAAGATCACTC 457
                                  *     *  *                **

L. acidophilus ATCC 4356 slpA  TAAAATTCACCGGTACAAA--CAGTGAT--AATCA--------------A 483
L. acidophilus ATCC 4356 slpB  CTGGTTTAGCTGATGCTAA--CAAGGTTTCAGCTACTTCA-------GAA 498
L. crispatus JCM 5810 cbsA     TGACTTTCCCTGAGGGTGA--CATGTTCAAGACTGC-------------T 495
L. crispatus MH315 lbsB        TGACTTTCCCTGAGGGTGA--CATGTTCAAGACCGC-------------T 477
L. crispatus LMG 12003 slpnB   TAACTTTCCCAGAAGGTGA--TGGCTTTAAGCTTGC-------------T 468
L. crispatus M247 s-layer      T---TGGCTCAAAGAACAG--CAATGTTAAATTTGCAG----------GT 492
L. crispatus MH315 lbsA        TTAAGCTTGCTAATGGTGA---ACTTTCAGGTAAGAA------------T 528
L. crispatus JCM 5810 cbsB     TTAAGTCATCAAACAGCAATGTAACTTTTGCATCATCAAACAGCAATGCT 468
L. crispatus LMG 12003 slpnA   TTGTTTCATCTAACTCAAAAGTAACTTTTGG------------------- 488
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L. acidophilus ATCC 4356 slpA  ACTGAAACTAATGTTTCTA---CTTTGAAAGTTAAGTTAGACCAAAACGG 530
L. acidophilus ATCC 4356 slpB  ACTTCAGTTGATGCAGGCAAGACTATCCAAGTTAAGACTGACAAGAACGG 548
L. crispatus JCM 5810 cbsA     ---GATACTTCTTTAGCACAATCACACGAAGTACAATTGGACAAGAACGG 542
L. crispatus MH315 lbsB        ---GATACTTCTTTAGCACAATCACACGAAGTAAAATTAGACCAAAACGG 524
L. crispatus LMG 12003 slpnB   TCAAACAACTCATTTACTAATTCAAGAACCATTCAACTTGACAAGAATGG 518
L. crispatus M247 s-layer      GCTGACGGTAAGTTTGCTGATACTGTAAAGGTTGAACTTGGTCAAAATGG 542
L. crispatus MH315 lbsA        GTGACTAAGAATACTGATGGTTCTTACAAGTTAACTTTGGACCAATATGG 578
L. crispatus JCM 5810 cbsB     CAAGTTTCAGCTGATGGTAAGACTGTTACTGCAACTTTAGACCAAAACGG 518
L. crispatus LMG 12003 slpnA   -----TTCAGATAATGCTAAGACTGTAACTGTTTCTTTAGACCAAAACGG 533
                                                *           *   *  * **

L. acidophilus ATCC 4356 slpA  TGTT---GCTTCACTTACTAATGTTTCAATTGCAAACGTATACGCAATTA 577
L. acidophilus ATCC 4356 slpB  TGTT---GTAAGCTTCGGTTCAGCACAAGTTCTTAACGTTAAGGTTGTTG 595
L. crispatus JCM 5810 cbsA     TACT---ACTACTTTACCAGAAGT---AGTTATGAACGTAACTGCTAAGA 586
L. crispatus MH315 lbsB        TACT---ATTACTTTGCCAGAAGT---AGTTATGAACGTAACTGCTAAGG 568
L. crispatus LMG 12003 slpnB   TACT---GTAACTTTAAATGAAGT---TGTATTACACGCAACTGCTAAGG 562
L. crispatus M247 s-layer      TACTTTAACTACCCCAATCTCAGTTCAAGTTTCAAACGTTAACGCACTTG 592
L. crispatus MH315 lbsA        TAACGCTACTGAATTGACTTACACTCAATCACTTAAGGCTTACAACCAAG 628
L. crispatus JCM 5810 cbsB     TACTGTTTCTGGCTTAACTGTTGT---TG--AAAGAT-TAGTTGCTTATG 562
L. crispatus LMG 12003 slpnA   TACAGCAAAGGACTTAACTGTTAA---CATCAGCGATGTAACTGCTTTCA 580
                               *                       *

L. acidophilus ATCC 4356 slpA  ACACTACTGATAACAGTAACGTAAACTTCTACGACGTAACTAGTGGTGCT 627
L. acidophilus ATCC 4356 slpB  AAACTAGCGACGTTAGAGCTGTTTCATTCTACGACATCCAAACTGGTAAG 645
L. crispatus JCM 5810 cbsA     ACTTTGCTAACCCAACTGTTGTTACTTGGTTGAATGGTACTACTTCAGCT 636
L. crispatus MH315 lbsB        ACTTCGCTAACCCAGCAGTAGTTAACTGGTACAACACTGCTACTAACGCA 618
L. crispatus LMG 12003 slpnB   ACTTTGCTAACCCAGCAGTTGTTAACTGGTACAATACTGCTACTAACTCA 612
L. crispatus M247 s-layer      ACCTTTCAAATGCTAACGGTGTTAACTTCTACAACGCTTCAAACGGTTCA 642
L. crispatus MH315 lbsA        GTAACACTAATTCTGTATTCTTTATTAACCAAAACAGTGGTACTA----- 673
L. crispatus JCM 5810 cbsB     ATGCAACTAATACTAATGATGTAGTATTCTACAACATTGCTACTGGTCAA 612
L. crispatus LMG 12003 slpnA   ACGCAACTAATACTAACGGTGTTGTATTCTACAACGTAACTACTGGTACA 630
                                     *           *       *       *

L. acidophilus ATCC 4356 slpA  ACTGTAACTAACGGTGCCGTTTCAGTTAATGCTGATAA---CCAAGGTCA 674
L. acidophilus ATCC 4356 slpB  ACTGTAGAAAACGGTACTCTTTCAATCGTTGCTGGTTC---TAACGCACG 692
L. crispatus JCM 5810 cbsA     CCTGTAACTGCTGGTAACATCACTTTATACGCTGGTTCAGATGCTGGCAA 686
L. crispatus MH315 lbsB        GTTGTAAGTACTGGTAACATTGAACTTTTCGCAGGTTCAGATGCTGGTAA 668
L. crispatus LMG 12003 slpnB   GTTGTAAGTACTGGTAACATTGAATTGTTTGCAGGTTCAGATGCTGGCAA 662
L. crispatus M247 s-layer      CAAGTAACTAAGGGTTCAGTTAATGTAACTGCTGGTCT---TATCGGTCG 689
L. crispatus MH315 lbsA        -CTGAAACCAAAGGTTTATACCTTACCCTTGCTAAT---GGCAATGGTGA 719
L. crispatus JCM 5810 cbsB     CCTGTAAATTCAGGCGATGCTATGGTTCTTGCTGATAG---CAACAAGCA 659
L. crispatus LMG 12003 slpnA   CAAGCACATGCTGGTAATGCCATGGTTCTTGCTAATAC---TCAAGGTCA 677
                                  * *      **              **   *

L. acidophilus ATCC 4356 slpA  AGTTAATGTTGCAAACGTAGTTGCAGCAATTAATTCAAAATACTTTGCAG 724
L. acidophilus ATCC 4356 slpB  TGCTAACGTACAAGAAATCGTTAACGCATTTAACGCTAAGTACCAAGCTT 742
L. crispatus JCM 5810 cbsA     GATGAACGTTGCTCAAGTTGTTGCAGAAGCAAGAAAGAATTATGTTGCTA 736
L. crispatus MH315 lbsB        GATGAACGTTGCTCAAGTTGTTTCAGCAACTGAAAAGAAGTACCACGCAA 718
L. crispatus LMG 12003 slpnB   GATGAACGTTGCTCAAGTTACTTCAGCTGCTTTAAAGAAGTACCACGCAA 712
L. crispatus M247 s-layer      TTTGAACGTTTCAACTGTTGCTAGTGAAATCTTAAAGAACTGTGCTGCTT 739
L. crispatus MH315 lbsA        ATTAAATGTTGCTGATGTTTTAGCTAATATTGAAAAGCAATACACTGCTG 769
L. crispatus JCM 5810 cbsB     ACTTAATGTTGCAGCTATCCTCCCAGCAGTTAAGAGTAACTTCACCGCTA 709
L. crispatus LMG 12003 slpnA   ACTTAACACTGCTGCACTTCTTCCTGCAATTGAAAGTAACTATGTAGCTG 727
                                  **          *                * *     **

L. acidophilus ATCC 4356 slpA  CACAAT----------------------ACGCAGATAAGAAGTT------ 746
L. acidophilus ATCC 4356 slpB  CTCAATTGAAC-----------A-----ACGCTAACAGCAATGC------ 770
L. crispatus JCM 5810 cbsA     TG--------------------G-----GTGCTAAGGTTG---------- 751
L. crispatus MH315 lbsB        GCAACTAC--------------G-----GTACTAAAGCTAACCA-AGAAT 748
L. crispatus LMG 12003 slpnB   GCAACTAC--------------G-----GTACTGCAGCTAACCA-AGAGT 742
L. crispatus M247 s-layer      AC---CAA--------------G-----TTTCAAATGGTAAGCC-CGTAT 766
L. crispatus MH315 lbsA        TTCAATACAAT-----------G-----ATTCAAAATTCATGAGTAGTAC 803
L. crispatus JCM 5810 cbsB     CCCAACGTGTA-ACAGTAGCTCAAGGTAACGGTAATGGTACTTACAGCCA 758
L. crispatus LMG 12003 slpnA   TTCAACGTGTAGATAGTGACA-GTGCTAATGGTAATGGTACTTACAACTT 776
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L. acidophilus ATCC 4356 slpA   ---AAATACTCGT---ACTGCTAATACT------GAAGATGCTATTAAGG 784
L. acidophilus ATCC 4356 slpB   ---TAACGTTCGTTTGACTGACAACAAC------GCTCAAGCTGTTGCTA 811
L. crispatus JCM 5810 cbsA      --------CTGACCCA---ACAAACA------------ACATCAAGGAAG 778
L. crispatus MH315 lbsB         CA--AGCACTATTTCATACACCAACA------------ACCTTAAGGATG 784
L. crispatus LMG 12003 slpnB    CA--AGCACTATTTCATACAGCAACA------------ACCTTGTTGAAG 778
L. crispatus M247 s-layer       CACAATTGCCAGACCAAAAGGCTGTAGT-----TGCTGATGTGAACGCTG 811
L. crispatus MH315 lbsA         TGAAAAGGATAGCCCAGTAACTATTACTACTAACAAGGATGCTGTAATTG 853
L. crispatus JCM 5810 cbsB      AGATCAAATTAACACTGTAAAGATCAATACTACTACTCCTGAAATTAAGG 808
L. crispatus LMG 12003 slpnA    TGCTGATTTTAAGCATGTAAATAATATTGAATTTGCCACTGCTATCAAGG 826
                                                  *

L. acidophilus ATCC 4356 slpA   CAGCCTTAAAGGACCAAAAGATTGATGTAAACTCAGTAGGTTACTTCAAA 834
L. acidophilus ATCC 4356 slpB   CTATGTTAAGAGCTCAAAACATTGATGTTGATGCACAAGGTTACTTCACT 861
L. crispatus JCM 5810 cbsA      CT---TTGAAGGCTATGAACATTGATGTTGATGCTCGAGGTTGGTTCGTT 825
L. crispatus MH315 lbsB         CT---TTAAAGGCTATGAACGTTGATGTTGATGCTCAAGGCTGGTTCGTT 831
L. crispatus LMG 12003 slpnB    CT---TTAAAGGCTGCTGGTGTTGAAGTTAAGGAT---AATTGGTTCGTT 822
L. crispatus M247 s-layer       CT---TTAAAGGCTGCTAATATTCCAGTTGACAATGCTGGGTGGTTTACT 858
L. crispatus MH315 lbsA         CTGAACTTAAGAAGCAAAACATCCCTGTTAATGCTGCTGGTAACTTCACT 903
L. crispatus JCM 5810 cbsB      ATCAATTAGAAAAGGCTGGCATTAAGATTGATGCTAACGGCAACTTTACT 858
L. crispatus LMG 12003 slpnA    ACCAATTGAAGGCTCAAAACATTGATGTAGGTCCTCAAGGTTTCTTCAAG 876
                                          *           *   *                **

L. acidophilus ATCC 4356 slpA   GCACCTCATACTTTCACTGTTAACGTTAAAGGAACTTCAAATACTAATGG 884
L. acidophilus ATCC 4356 slpB   GCACCAGCTTCATTGAGCTTAACTTTCCACGCAGAATCAACTCAAAACAA 911
L. crispatus JCM 5810 cbsA      GCTCCTAAGTCATTTACTTTCAACTTGACTGCTAAGTCAGACGTAAATGA 875
L. crispatus MH315 lbsB         GCTCCTAAGTCATTCACTTTCAACATGACTGCTAAAGCTAACAACAATGA 881
L. crispatus LMG 12003 slpnB    GCACCTAAGTCATTTACTTTCAACATGACTGCAACTGCTAACAACAACGA 872
L. crispatus M247 s-layer       GCTCCAATCTCATTATCAGTTAATGTAAAGGCAAGTTCATCAATTAATGG 908
L. crispatus MH315 lbsA         GCTCCTGACACCTTCACTGTGACTTTGAACGCTAAGTCAAGCATCAACGG 953
L. crispatus JCM 5810 cbsB      GCACCTCACTCATTTAAGGTAACTGTAAAGGCTACTTCAGACGTTAACGG 908
L. crispatus LMG 12003 slpnA    GCACCTCATACATTTACTGTTAAGGTTAAGGCAACTTCAAGCATTAACGG 926
                                ** **     * **    * *   *    **     *        **

L. acidophilus ATCC 4356 slpA   TAAGTCA-GCTACTTTGCCAGTAGTTGTTACTGTTCCTAATG-------- 925
L. acidophilus ATCC 4356 slpB   TGAAACT-GCACAATTACCAGTAACTGTTTCAGTAACTAACGGTAAGGAA 960
L. crispatus JCM 5810 cbsA      TGCTACT-GCAACTTTACCAGTAACTGTTAACGTACCAAACGG------- 917
L. crispatus MH315 lbsB         TGCTTCA-AGTACCTTAGCTGTAACTGTTTCAGTTCCAAACGG------- 923
L. crispatus LMG 12003 slpnB    TGCTTCA-AAGACTTTAGCTGTAACTGTTAGCGTACCAAACGG------- 914
L. crispatus M247 s-layer       TGTTGGATGTTACTTTACCTGTA-CTGTTAATGTTGCTAACGG------- 950
L. crispatus MH315 lbsA         CAAGACTGGTCAA-TTAGTAGTAACTGTTTCAGTTCCAAACGG------- 995
L. crispatus JCM 5810 cbsB      CAAGAGC-AAGGAATTACCTGTAACTTTCACTGTAGCTAACGT------- 950
L. crispatus LMG 12003 slpnA    TAAGAGT-GAAGAACTTCCCGTAACCTTTACTGTAGCTAACGT------- 968
                                        *   ***   *   **  * ** *

L. acidophilus ATCC 4356 slpA   -TTGCTGAGCCAACTGTAGCCAGCGTAAGCAAGAGAATTATGCACAACGC 974
L. acidophilus ATCC 4356 slpB   GTTACTCCTTCAACTGTAGACAGCGTAAGCAAGAGTTTTATGCACAATGC 1010
L. crispatus JCM 5810 cbsA      --CAAGGACACTACTGTACCAAGCCAAAGCAAGACTGTTATGCACAACGC 965
L. crispatus MH315 lbsB         --TAAGGACATGACTGTACCAAGCCAAAGCAAGACTGTTATGCACAACGC 971
L. crispatus LMG 12003 slpnB    --CAAGGACATGACTGTACCAAGCCAAAGCAAGACTATTATGCACAACGC 962
L. crispatus M247 s-layer       --CAAGGACATGACTGTACCAAGCCAAAGCAAGACTATTATGCACAATGC 998
L. crispatus MH315 lbsA         --TAAGAAGACTACTGTTGCTAGCCAAGAAAAGACTATTATGCACAACGC 1043
L. crispatus JCM 5810 cbsB      --TGCAGAACCAACTGTTGCTAGCCAAAGCAAGATGATTATGCACAACGC 998
L. crispatus LMG 12003 slpnA    --TGCAGATCCTGTTGTTCCAAGTCAACCTAAGACTATTATGCACAACGC 1016
                                      ***    **   *   ****   ********** **

L. acidophilus ATCC 4356 slpA   ATACTACTACGACAAGGACG---CTAAGCGTGTTGGTACTGACAGCGTTA 1021
L. acidophilus ATCC 4356 slpB   ATACTACTACGACAAGGACG---CTAAGCGTGTTGGTACTGACAGCGTTA 1057
L. crispatus JCM 5810 cbsA      TTACTTCTACGACAAGAACG---GCAAGCGCGTTGGTTCTGACAAGGTAA 1012
L. crispatus MH315 lbsB         ATTCTTCTATGACAAGAACG---GCAAGCGTGTTGGTTCTGACAAGGTAA 1018
L. crispatus LMG 12003 slpnB    ATTCTACTACGACAAGAACG---GCAAGCGTGTTGGTTCTGACAAGGTAA 1009
L. crispatus M247 s-layer       ATACTACTACGACAAGGACG---CTAAGCGTGTTGGTACTGACAAGCTTA 1045
L. crispatus MH315 lbsA         ATATTACTACGACAAGGATG---CTAAGCGTGTTGGTACTGACAAGGTAA 1090
L. crispatus JCM 5810 cbsB      TTACTACTACAAGGAAGACGGTACTACTCGTGCTAACAACGACAAGGCTA 1048
L. crispatus LMG 12003 slpnA    ATACTACTACAAGGAAGATGGTACTACTCGTGCCAACAACGACAAGGCTA 1066
                                * * *** * * * *   * ** *       ****     *
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A.

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L. acidophilus ATCC 4356 slpA  AGCGTTACAACTCAGTAAGCGTATTGCCAAACACTACTACTATCAACGGT 1071
L. acidophilus ATCC 4356 slpB  AGCGTTACAACTCAGTAAGCGTATTGCCAAACACTACTACTATCAACGGT 1107
L. crispatus JCM 5810 cbsA     CTCGTTACAACTCAGCAACTGTTGCTATGTCAACTACTACCATCAAGGGC 1062
L. crispatus MH315 lbsB        CTCGTTACAACTCAGCAACTGTTGCTATGAATACTACTACTATCAACGGC 1068
L. crispatus LMG 12003 slpnB   CTCGTTACAACTCAGCAACTGTTGCTATGAATACTACTACTATCAACGGC 1059
L. crispatus M247 s-layer      CCCGTTACAACTCAGTAACTGTTGCTATGAACACTACTACTATCAACGGC 1095
L. crispatus MH315 lbsA        CTCGTTACAACAAGGTAACTGTTGCAACTTCAACTACTAAGATCGGTGAC 1140
L. crispatus JCM 5810 cbsB     AGCGTTACGAATCAGTAACTGTTGCTATGTCAACTAAGAAGATTGGCGAC 1098
L. crispatus LMG 12003 slpnA   AGCGTTATGAATCAGTAAATGTTGCTATGTCAACTAAGAAGATCGGCAAC 1116
                                 *****  *   * **  **          ****  *  **

L. acidophilus ATCC 4356 slpA  AAGACTTACTACCAAGTAGTTGAAAACGGTAAGGCTGTTGACAAGTACAT 1121
L. acidophilus ATCC 4356 slpB  AAGGCTTACTACCAAGTAGTTGAAAACGGCAAGGCAGTTGACAAGTACAT 1157
L. crispatus JCM 5810 cbsA     AAGGCTTACTACGAAGTAATCGAAAACGGTAAGGCTACTGGTAAGTTCAT 1112
L. crispatus MH315 lbsB        AAGGCTTACTACGAAGTAATCGAAAACGGTAAGGCTACTGGTAAGTTCAT 1118
L. crispatus LMG 12003 slpnB   AAGGCTTACTACGAAGTAATCGAAAGCGGTAAGGCTACTGGTAAGTTCAT 1109
L. crispatus M247 s-layer      AAGGCTTACTACGAAGTAATCGAAAATGGTAAGGCTACTGGCAAGTTCAT 1145
L. crispatus MH315 lbsA        AAGACTTACTACGAAGTAATCGAAAACGGCAAGGCTACTGGCAAGTACAT 1190
L. crispatus JCM 5810 cbsB     AAGAACTTCTACGAAGTAATTAAGGACGGCAAGGCTACTAGTATGTACAT 1148
L. crispatus LMG 12003 slpnA   AAGGACTTCTATGAAGTAATCAAGGATGGCAAGGCTACTGGTATGTACAT 1166
                                 ***   * ***  ***** * *  *    ** *****   *   * ** ***

L. acidophilus ATCC 4356 slpA  CAACGCTGCAAACATCGATGGTACTAAGCGTACTTTGAAGCACAACGCTT 1171
L. acidophilus ATCC 4356 slpB  CAACGCTGCAAACATCGATGGTACTAAGCGTACTTTGAAGCACAACGCTT 1207
L. crispatus JCM 5810 cbsA     CAACGCTGCCAACATTGATGGTACTAAGCGTACTTTGAAGCACAACGCAT 1162
L. crispatus MH315 lbsB        CAACGCTGCCAACATTGATGGTACTAAGCGTACTTTGAAGCATAACGCAT 1168
L. crispatus LMG 12003 slpnB   CAACGCTGCCAACATTGATGGTACTAAGCGTACTTTGAAGCACAACGCAT 1159
L. crispatus M247 s-layer      CAACGCAGACAACATTGATGGTACTAAGCGTACTTTGAAGCACAACGCAT 1195
L. crispatus MH315 lbsA        CAACGCCGACAACATCGACGGTACTAAGCGTACTTTGAAGCACAACGCAT 1240
L. crispatus JCM 5810 cbsB     CAACGCTGACAACATCGATGGTACTAAGCGTACTTTGAAGCACAACGCAT 1198
L. crispatus LMG 12003 slpnA   CAACGCTGACAACATTGACGGTACTAAGCGTACTTTGAAGCACAACGCAT 1216
                               ******* *  ***** ** ************************ ***** *

L. acidophilus ATCC 4356 slpA  ACGTTTACGCATCATCAAAGAAGCGTGCTAACAAGGTTGTATTGAAGAAG 1221
L. acidophilus ATCC 4356 slpB  ACGTTTACGCATCATCAAAGAAACGTGCTAACAAGGTTGTATTGAAGAAG 1257
L. crispatus JCM 5810 cbsA     ACGTTTACAAGTCTTCAAAGAAGCGTGCTAACAAGGTTGTTCTTAAGAAG 1212
L. crispatus MH315 lbsB        ACGTTTACAAGTCTTCAAAGAAGCGTGCTAACAAGGTTGTTCTTAAGAAG 1218
L. crispatus LMG 12003 slpnB   ACGTTTACAAGTCTTCAAAGAAGCGTGCTAACAAGGTTGTTCTTAAGAAG 1209
L. crispatus M247 s-layer      ACGTTTACAAGACTTCAAAGAAGCGTGCTAACAAGGTTACCTTGAAGAAG 1245
L. crispatus MH315 lbsA        ACGTTTACGCAACTTCAAAGAAGCGTGCTAACAAGTTTGTTCTTAAGAAG 1290
L. crispatus JCM 5810 cbsB     ACGTTTACAAGACTTCAAAGAAGCGTGCTAACAAGGTTGTTCTTAAGAAG 1248
L. crispatus LMG 12003 slpnA   ACGTTTACAAGACTTCAAAGAAGCGTGCTAACAAGGTTGTTCTTAAGAAG 1266
                               ********     * ******** ************ **     * ******

L. acidophilus ATCC 4356 slpA  GGTGAAGTTGTAACTACTTACGGTGCTTCATACACATTCAAGAACGGCCA 1271
L. acidophilus ATCC 4356 slpB  GGTGAAGTTGTAACTACTTACGGTGCTTCATACACATTCAAGAACGGCCA 1307
L. crispatus JCM 5810 cbsA     GGTACTGAAGTAACTACTTACGGTGGTGCTTACACCTTCAAGAACGGCAA 1262
L. crispatus MH315 lbsB        GGTACTGAAGTAGTTACCTACGGTGGTGCTTACACCTTCAAGAACGGCAA 1268
L. crispatus LMG 12003 slpnB   GGTGACACTGTTGTCACCTACGGTGGTACTTACACATTCAAGAACGGCAA 1259
L. crispatus M247 s-layer      GGTACTGAAGTAACTACTTACGGTGGTACTTACACATTCAAGAACGGCAA 1295
L. crispatus MH315 lbsA        GGTGAAGAAGTAACTACTTACGGTGGTACTTACACATTCAAGAACGGCAA 1340
L. crispatus JCM 5810 cbsB     GGTGAAGAAGTAACTACTTACGGTGGTACTTACACATTCAAGAACGGCAA 1298
L. crispatus LMG 12003 slpnA   GGTGACACTGTTGTTACCTACGGTGGTACTTACACATTCAAGAACGGCAA 1316
                               ***      **    ** ******* * * ***** ************ *

L. acidophilus ATCC 4356 slpA  AAAGTACTACAAGATCGGTGACAACACTGACAAGACTTACGTTAAGGTTG 1321
L. acidophilus ATCC 4356 slpB  AAAGTACTACAAGATCGGTGACAACACTGACAAGACTTACGTTAAGGTTG 1357
L. crispatus JCM 5810 cbsA     GCAATACTACAAGATCGGTAACAACACTGACAAGACTTACGTAAAGGCTT 1312
L. crispatus MH315 lbsB        GCAATACTACAAGATCGGTAACAATACTGACAAGACTTACGTAAAGGCTT 1318
L. crispatus LMG 12003 slpnB   GCAATACTACAAGATCTACAACAATACTGAAAAGACTTACGTAAAGGCTT 1309
L. crispatus M247 s-layer      GCAATACTACAAGATCGGTAACAACACTGACAAGACTTACGTAAAGGCTT 1345
L. crispatus MH315 lbsA        GCAATACTACAAGATCGGCAACGATACTAAGAAGACTTACGTAAAGGCTT 1390
L. crispatus JCM 5810 cbsB     GCAATACTACAAGATCGGCAACGATACTAAGAAGACTTACGTAAAGGCTT 1348
L. crispatus LMG 12003 slpnA   GCAATACTACAAGATCTACAACAATACTGAAAAGACTTACGTAAAGGCTT 1366
                                 * *************     ** * *** * ************ **** *
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A.

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L. acidophilus ATCC 4356 slpA   CAAACTTTAGATAA- 1335
L. acidophilus ATCC 4356 slpB   CAAACTTTAGATAA- 1371
L. crispatus JCM 5810 cbsA      CAAACTTTTAA---- 1323
L. crispatus MH315 lbsB         CAAACTTTTAA---- 1329
L. crispatus LMG 12003 slpnB    CAAACTTTTAA---- 1320
L. crispatus M247 s-layer       CAAACTTTTAA---- 1356
L. crispatus MH315 lbsA         CAAACTTTTAA---- 1401
L. crispatus JCM 5810 cbsB      CAAACTTTTAATTAA 1363
L. crispatus LMG 12003 slpnA    CAAACTTTTAA---- 1377
                                ********  *
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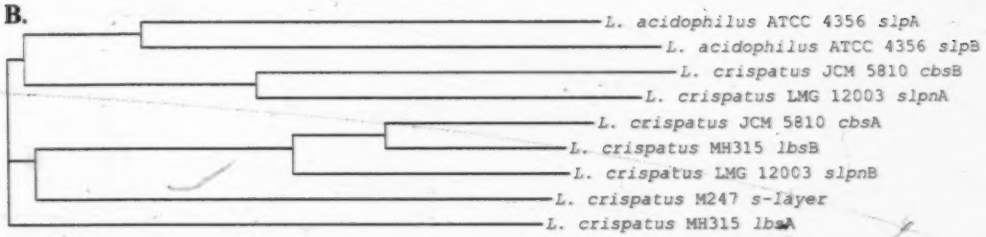
B.


L. acidophilus ATCC 4356 slpA
L. acidophilus ATCC 4356 slpB
L. crispatus JCM 5810 cbsB
L. crispatus LMG 12003 slpnA
L. crispatus JCM 5810 cbsA
L. crispatus MH315 lbsB
L. crispatus LMG 12003 slpnB
L. crispatus M247 s-layer
L. crispatus MH315 lbsA

C.

	L. acidophilus ATCC 4356 *slpA*	*L. acidophilus* ATCC 4356 *slpB*	*L. crispatus* JCM 5810 *cbsA*	*L. crispatus* JCM 5810 *cbsB*	*L. crispatus* LMG 12003 *slpnA*	*L. crispatus* LMG 12003 *slpnB*	*L. crispatus* M247 *s-layer*	*L. crispatus* MH315 *lbsA*	*L. crispatus* MH315 *lbsB*
L. acidophilus ATCC 4356 *slpA*		72	65	66	67	64	69	68	65
L. acidophilus ATCC 4356 *slpB*			67	64	63	67	67	66	66
L. crispatus JCM 5810 *cbsA*				64	67	82	70	69	89
L. crispatus JCM 5810 *cbsB*					77	64	65	66	66
L. crispatus LMG 12003 *slpnA*						68	66	68	66
L. crispatus LMG 12003 *slpnB*							70	70	86
L. crispatus M247 *s-layer*								69	70
L. crispatus MH315 *lbsA*									69
L. crispatus MH315 *lbsB*									

predicted to encode a 24 amino acid N-terminal signal sequence, although this was later corrected to be a 31 amino acid signal peptide based on mass spectrometry data of the mature protein (Fig. 1.3A) [9].

Southern analysis of the *L. acidophilus* ATCC 4356 genomic DNA with the *slp*A probe identified a second gene, *slp*B [9]. The gene *slp*B consists of an open reading frame of 1368 nucleotides, corresponding to a 456 amino acid protein with a predicted molecular weight of 47.7 kDa (Fig. 1.3A). Comparison of *slp*A, *slp*B, and flanking sequence identified the following similarities: the 185 nt immediately upstream of the gene (Fig. 1.4), the 5' end of the gene (1-95 nt), and the 3' end (nt 1220 to 1648), with reduced homology in the internal sequence and no homology in DNA in further upstream or downstream sequences aside from weak similarity of the terminators (Figs. 1.3, 1.4 & 1.5).

Northern analysis revealed that the *slp*A locus encodes the gene responsible for the S-protein that is expressed *in vitro* (i.e. SlpA, the first *L. acidophilus* S-protein studied) [9]. The promoter for *slp*A was located upstream of the gene in the 5' untranslated region of similarity between *slp*A and *slp*B. Further research revealed two promoter sequences upstream of *slp*A (starting at –335 nt and –228 nt from the *slp*A start codon [i.e. 335 and 228 nt upstream of the start codon]), however only P1, the closer promoter, appeared to be active in laboratory culture [10]. No promoters were associated with *slp*B [9]. A ribosome binding site was found 9 nt upstream from the start codon. Both *slp*A and *slp*B genes end in two stop codons, which were predicted to be necessary for efficient termination [8,9]. A Rho-independent terminator was also found downstream of both ORFs.

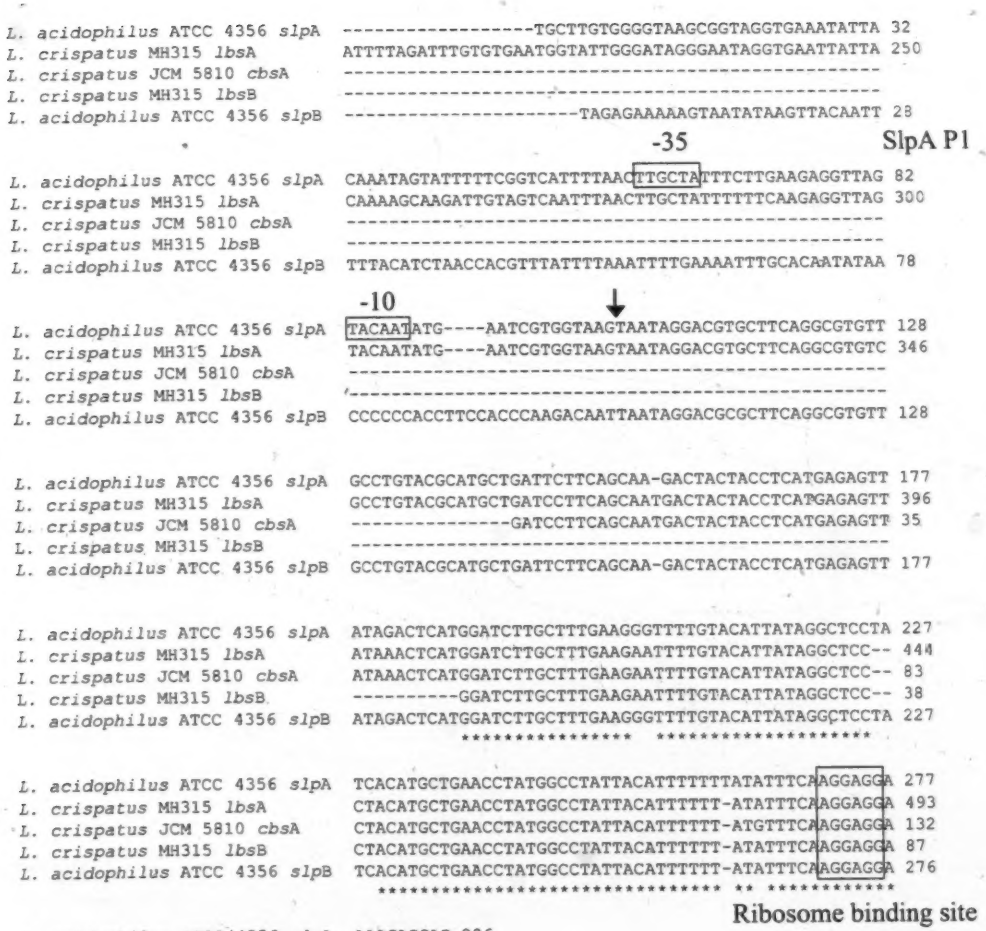


Fig. 1.4. Comparison (ClustalW alignment) of upstream region of group A *acidophilus* S-protein genes. Boxes indicate SlpA promoter sequence and ribosome binding site, as labeled. "*" indicates identical nucleotide in all sequences. Arrow indicates the start of homologous sequence between the upstream regions of *slp*A and *slp*B. The GenBank accession numbers are as follows: ATCC 4356 *slp*A, X89375; ATCC 4356 *slp*B, X89376; JCM 5810 *cbs*A, AF001313; MH315 *lbs*A (partial), AB110090; MH315 *lbs*B, AB110091.

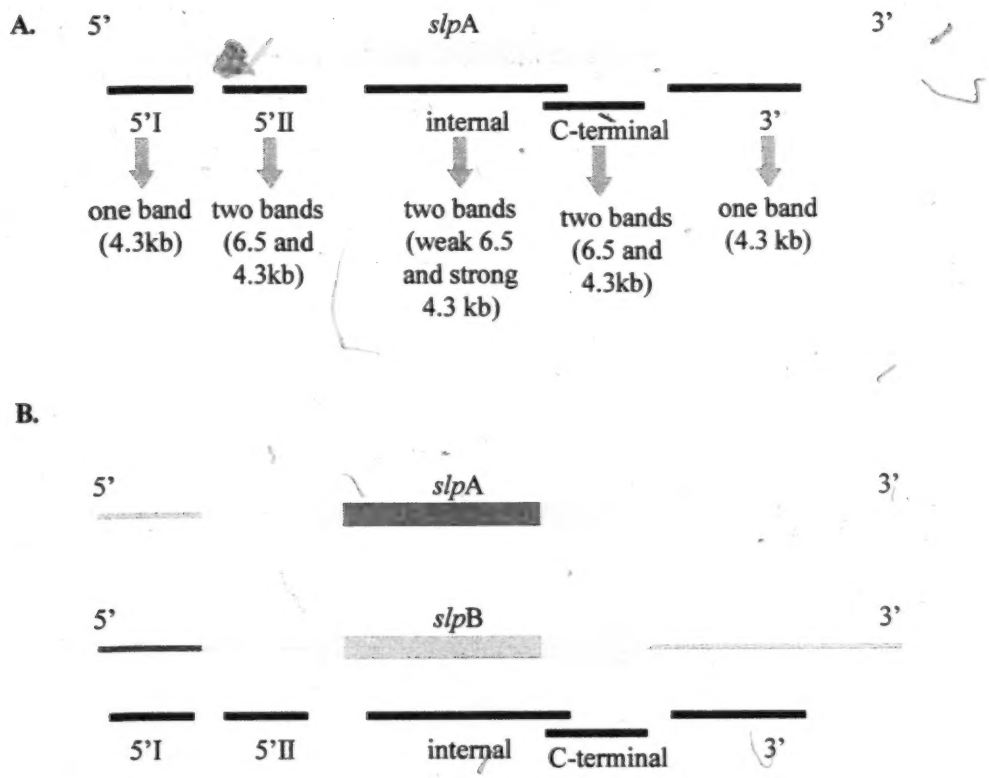


Fig. 1.5. Homology between *slp*A and *slp*B in *L. acidophilus* ATCC 4356. **A.** Results obtained from probes used to detect *slp* regions with Southern analysis. Modified from Boot et al [9]. **B.** Interpretation of results from Southern analysis. Different shades represent low homology.

The half-life of the mRNA transcript from *slp*A was found to be 15 minutes, and the predicted secondary structure of the mRNA featured a 5' end which is unavailable for degradation [10]. However, the ribosome binding site is exposed in a stem loop in this predicted structure, allowing transcription using the stable, folded mRNA template. The upstream untranslated region is essential for the formation of the stable secondary mRNA structure. The activity of the promoter was determined using a reporter system, and was found to be very high when compared to the *L. casei* LDH promoter [49], when expressed in *L. casei* ATCC 393 [10]*. Taken together, the high promoter activity and the stability of the mRNA of the *slp*A gene may account for the high predicted rate of S-protein production in exponentially growing cells (*ca.* 500 subunits synthesized per second for cells with a generation time of 20 minutes [83]). This promoter could have useful applications for overexpression of proteins in lactobacilli. The double stop codons and terminator are likely required for efficient regulation of such a highly transcribed gene.

In addition to imparting stability to the mRNA of *slp*A, regions of homology between both genes upstream of the start codon were also predicted to be involved in chromosomal rearrangements resulting in expression of the *slp*B via the active (P1) promoter (Fig. 1.6) [9]. This was confirmed in 1996 [12]. Using Northern analysis, Boot et al. [9] determined that the majority of the transcripts from *in vitro* cultures were from *slp*A, thereby designating *slp*B as silent. The low but detectable production of *slp*B mRNA was suggested to come from a minority of isolates in the culture expressing SlpB

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* The same strain of *L. casei* was used in a number of experiments discussed in this Chapter. It was later typed as *L. zeae* by Tynkkynen et al. [97] in 1999.

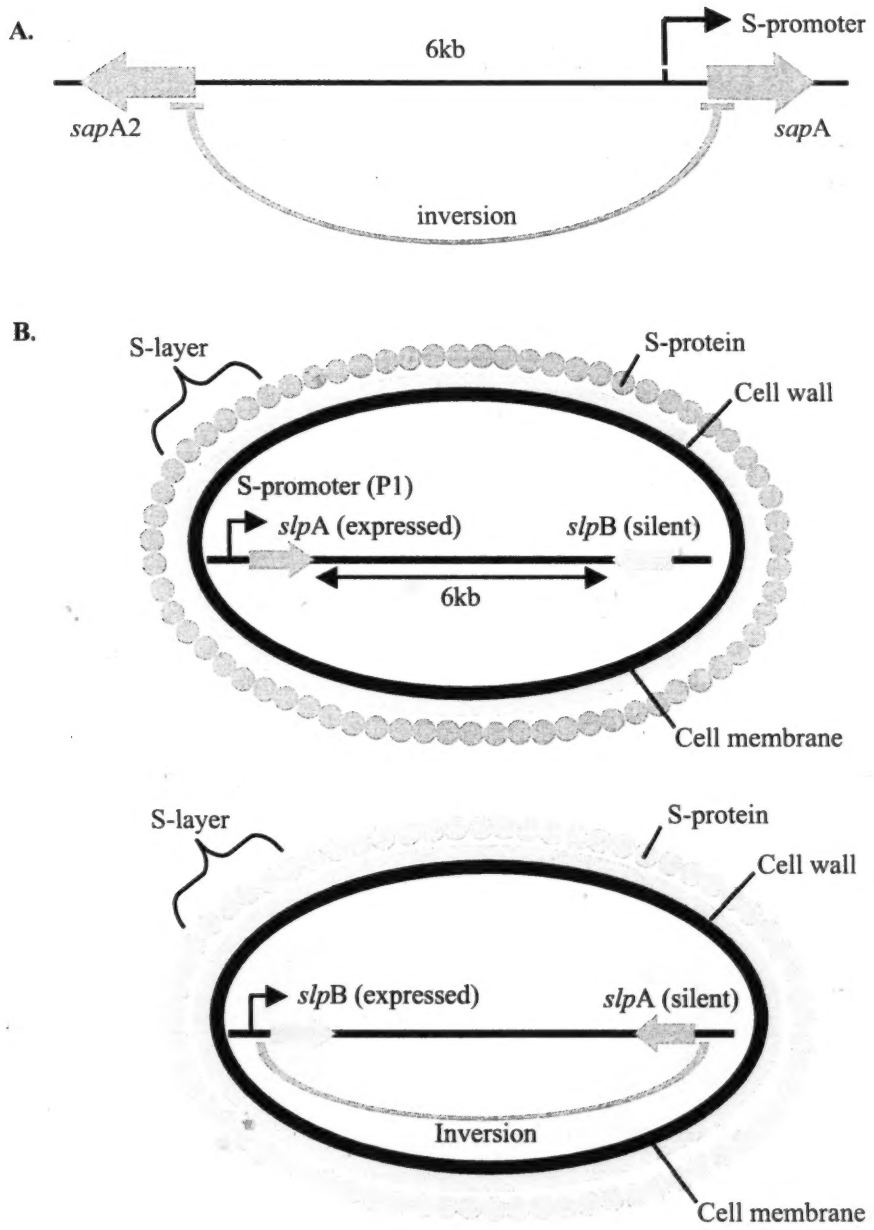

Fig. 1.6. Mechanisms of S-protein variation in gastrointestinal bacteria. Genetic rearrangements resulting in differential expression of S-proteins in **A.** *Campylobacter fetus* and **B.** *Lactobacillus acidophilus* ATCC 4356. Modified from Boot et al [13].

rather than SlpA. However, the detection of transcribed *slp*B mRNA from *L. acidophilus* isolates was not correlated with detection of SlpB by anti-SlpB antibodies [12]. The authors proposed that the failure to detect the SlpB may be due to a disadvantage in growth and survival of the SlpB producing strains. Further, they found that inactivation of *slp*A failed to produce viable mutants, suggesting that in *L. acidophilus* grown under laboratory conditions, *slp*A expression is essential.

Boot et al. [11] screened the type strains and one non-type strain of each of the GAA species for the presence of S-protein genes using Southern analysis. The non-type strains of *L. acidophilus*, *L. crispatus*, and *L. gallinarum* were of human and fowl intestinal origin. Four probes were tested: a 5' probe (nt –146 to +179 of *slp*A [i.e. from 146 nt upstream of the start codon to 179 nt downstream of the start codon] which includes the homologous recombination region [12]), a 3' probe (nt +1017 to +1386 of *slp*A), an *slp*A specific probe (nt +179 to +352 of *slp*A), and an *slp*B specific probe (nt +175 to +324 of *slp*B). Two fragments hybridized to the 5' and 3' probes in both strains of *L. crispatus*, *L. amylovorus*, and *L. gallinarum* strains tested. Hybridizations with the *slp*A and *slp*B specific probes indicated that one *slp*A homologue was present in the non-type strain of *L. crispatus* and both strains of *L. amylovorus*. Intense bands were only observed for the *slp*B probe in *L. acidophilus* strains. Based on these results, it can be determined that both genes in the strains studied contained the homologous regions directly upstream of the genes [11] which could be involved in gene recombination resulting in S-layer variation as found in *L. acidophilus* ATCC 4356 [12], as well as conserved 3' ends [11]. The *slp*A and *slp*B specific probes indicate that unique sequences within these S-protein genes are not conserved between all GAA members.

This data is supported by Western analysis of S-layers extracted from type strains and natural isolates: polyclonal mouse antibodies directed against *L. acidophilus* SlpA protein showed a strong reaction *with L. amylovorus*, while a weaker reaction was observed for *L. gallinarum* and no reaction for *L. crispatus* [11]. No further information has been published regarding S-proteins in *L. amylovorus* or *L. gallinarum*.

Since the analysis and sequences of S-protein genes in *L. acidophilus* ATCC 4356 were published, the S-protein genes in *L. crispatus* have been investigated. In 2000, Sillanpää et al. [81] published the sequence of *cbs*A, an S-protein gene in *L . crispatus* JCM 5810. They purified and sequenced S-protein peptide fragments from the 43 kDa protein on SDS-PAGE and also obtained the N-terminal sequence of the protein. This information was then used to design degenerate probes to detect part of the S-protein gene by Southern hybridization, which was cloned into *E. coli* and sequenced. The 3' end of the *cbs*A gene was also cloned and sequenced. Taken together, this information was used to assemble the *cbs*A ORF of 1317 nt, encoding a 440 amino acid protein with a 30 amino acid signal sequence (Fig. 1.3A). The second S-protein gene was detected in *L. crispatus* JCM 5810 by PCR-amplifying chromosomal DNA with primers in the N-terminal signal sequence and in the 3' conserved region of *cbs*A. The *cbs*B gene was inserted into pUC19 and cloned in *E. coli*. An ORF of 1359 nt, encoding a mature protein of 429 amino acids (452 aa including the signal sequence) was sequenced (Fig. 1.3A). Transcriptional activity of *cbs*A and *cbs*B were investigated by Northern blotting, and only the *cbs*A probe gave a positive reaction, indicating that *cbs*A is expressed *in vitro*. Aside from the detection of a putative ribosome binding site for *cbs*A, no analysis of the upstream and downstream regions of the *L. crispatus* S-protein genes has been described.

Therefore, no investigation regarding the regulation and expression of these genes has been published, although it stands to reason that the homologous recombination region seen in *L. acidophilus* ATCC 4356 [9] could be present in *L. crispatus* JCM 5810 as well. S-protein genes (*slpn*A and *slpn*B) have been identified and sequenced in *L. crispatus* LMG 12003, although this information has not been published, aside from a GenBank entry. Sillanpää et al. [81] state that *slpn*B is silent in laboratory culture conditions, thereby implying that *slpn*A is expressed *in vitro*.

1.3.2.3. Structural and Functional analysis of group A *acidophilus* S-proteins

Based on comparisons between predicted and observed molecular weights, Boot et al. [9] concluded that the SlpA protein is not glycosylated. This data is supported by Greene and Klaenhammer [36]. However, Möschl et al. [68] found that two strains of *Lactobacillus* (*L. plantarum* 41021/252 and *L. buchneri* 41021/251) do contain glycosylated S-proteins, although these are the first and only reported *Lactobacillus* glycoproteins to date.

Boot et al. [9] suggested that similarity in the predicted protein C-terminal sections of SlpA and SlpB may be an essential structure of the S-protein and may function as an attachment site of the S-layer to the cell wall and/or play a role in S-layer assembly, while the less homologous sequences in the middle may provide different antigens or different adhering effects on host tissues. The recent work by Smit et al. [84-86] is the first comprehensive attempt to functionally map the S-protein of *L. acidophilus* ATCC 4356, and ultimately confirmed the prediction regarding C-terminal cell wall anchoring and N-terminal functions. Smit et al. [84] divided the mature SlpA protein into two functionally separate domains: the N-terminal 2/3 of the protein (~290 amino acids)

named SAN, and the conserved C-terminal 1/3 of the protein (~123 amino acids) named SAC (Fig. 1.7A & D). *E. coli* clones containing and expressing these two domains were used to demonstrate their roles. SAC was cloned and expressed in *E. coli* but could be expressed only as a GFP-SAC fusion. Purified GFP-SAC bound to *L. acidophilus*, *L. crispatus*, and *L. helveticus* cells that were stripped of their native S-layer using 5M LiCl. SAC is highly hydrophilic and strongly positively charged and is therefore hypothesized to interact with the negative phospholipid head charges on lipid membranes. Indeed, the outer surface of an artificial S-layer extracted from *L. acidophilus* was found to be hydrophobic and the inner surface hydrophilic, implying the same situation when the S-layer is on the cell thereby making the outer surface of the cell with an in-tact S-layer hydrophobic. Further, SAC could be proteolytically cleaved from the LiCl extracted SlpA, but not from SlpA located on *Lactobacillus* cells, suggesting the C-terminus is protected and likely below the outer surface of the S-layer. Amino acid sequence analysis suggested the C-terminus is homologous to carbohydrate binding regions of other Gram-positive extracellular proteins, thereby suggesting the C-terminal region of the SlpA may anchor the S-layer to the cell by binding to a carbohydrate moiety.

When SAN was expressed in *E. coli*, purified from inclusion bodies and renatured, it formed crystals with a lattice identical to that of native S-proteins from *L. acidophilus*, suggesting that the N-terminal region is involved in S-layer crystallization [84]. Analysis of the structure and function of SAN was conducted using scanning mutagenesis, epitope insertions and protease sensitivity [85]. These analyses indicated the

A.

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L. crispatus LMG 12003 SlpnB   MKRNLRIVSAAAAALLAVAPVAASAVSVNAD---AVQSATQ--LG----- 40
L. crispatus MH315 LbsB        MKKNLRIVSAAAAALLAVAPVAASAVSVNAASSSAVQTATN--IG----- 43
L. crispatus JCM 5810 CbsA     MKKNLRIVSAAAAALLAVAPVAASAVSVNAD---AVSSANNSNLGNNNNG 47
L. crispatus M247 S-layer      MKKNLRIVSAAAAALLAVAPVAASAVSVNAADNTVATTTNTANTVINADG 50
L. crispatus JCM 5810 CbsB     -------VSAPAAALLAVAPVAASAVSVNAADSTATTTAKATDYTNINLG 43
L. crispatus LMG 12003 SlpnA   MKKNLRIVSAAAAALLAVAPVAASAVSVNAADSTATTTANATDYTNINLG 50
L. crispatus MH315 LbsA        MKKNLRIVSAAAAALLAVAPVAASAVSTVSADAAATTTATTNSNVTLNLN 50
L. acidophilus ATCC 4356 SlpA MKKNLRIVSAAAAALLAVAPVAASAVSTVSAATTINASSSA--------- 41
L. acidophilus ATCC 4356 SlpB MKKNLRIVS-AAAALLAVAPVAASAVSTVNAAAVNAIAVGG--------- 40
                                ** .*****************.          :

L. crispatus LMG 12003 SlpnB   --TVPALSNGDTVNVKPNVSLNTSA--YEG---VKANISVSFSATVNGTT 83
L. crispatus MH315 LbsB        --TVLPLTDGSTVNVKPNISLNTSA--YEG---VKANISVSFSATVDGTT 86
L. crispatus JCM 5810 CbsA     TFTVLPLNNGATVNVKPNISLNTSA--YEG---VKANISVSFSATVDGTT 92
L. crispatus M247 S-layer      TAINTPADAKYDVDVTPNLTATAAS--TVNGQTINGSITGNITASYNGQS 98
L. crispatus JCM 5810 CbsB     --GSAVSNNENQVDVTPALTLNG----TKGN--IKASLTGSITASFGGKS 85
L. crispatus LMG 12003 SlpnA   --GTTVSNTENQVDVTPSIVLNGNVKNTAGNLISKATLSGSISATFGGKS 98
L. crispatus MH315 LbsA        GAGSTATDAANTVNVSSNFSLNAPVK-ANNAVTADATLGGELTATLNGTS 99
L. acidophilus ATCC 4356 SlpA --INTNTNAKYDVDVTPSVSAVAANT-ANNTPAIAGNLTGTISASYNGKT 88
L. acidophilus ATCC 4356 SlpB --SATPLPNNSDVQISSSVAGVTTK---NGSSYTNGRISGSINASYNGTS 85
                                *::.. .          .    . :   :.*: .* :

L. crispatus LMG 12003 SlpnB   AVSNFKPGASEISLWKVEKD----KVTQVTDLQKVTSSNAGATYQVKMTN 129
L. crispatus MH315 LbsB        ATSNFTPNASTIELWKNEKD----KVTQVTDLQQVTSSNAGATYQVKMTQ 132
L. crispatus JCM 5810 CbsA     ATSNFTPNASTIELWKNEKD----KVTQVTDLQQVTSSNAGATYQVKMTQ 138
L. crispatus M247 S-layer      YTGTLDTKNGKVSVADS-------KGTAVTDFSKLTNG----SYTVTVSG 137
L. crispatus JCM 5810 CbsB     FTANLTGTEQNNVTINGNA-----------AKDELANVNAGDTVTVSVAN 124
L. crispatus LMG 12003 SlpnA   YTANLRGTDQNNVLINGRT-----------AKDELSNVNAGSSNTITIKN 137
L. crispatus MH315 LbsA        VSSSLADAAQDVTVSDGKTNLYSYNKETKKVENNLNNVVAGQSYTLTLTN 149
L. acidophilus ATCC 4356 SlpA YTANLKADTENATITAAGST--------TAVKP--AELAAGVAYTVTVND 128
L. acidophilus ATCC 4356 SlpB YSANFSSSNAGVVVSTPGHT--------ELSGEQINGLEPGSAVTVTLRD 127
                                ..:                                  :  :.:

L. crispatus LMG 12003 SlpnB   -VGLNFGSQNANKKITLTFPE--GDGFKLASNN---------SFTNSRTI 167
L. crispatus MH315 LbsB        -VGLNFGSQNANKKVTLTFPE--GDMFKTADT----------SLAQSHEV 169
L. crispatus JCM 5810 CbsA     -VGLNFGSQNANKKVTLTFPE--GDMFKTADT----------SLAQSHEV 175
L. crispatus M247 S-layer      -VSFNFGTANANKTITLGSKN--SNVKFAGADG---------KFADTVKV 175
L. crispatus JCM 5810 CbsB     -VGFNFGSENKGKKVTFKSSN--SNVTFASSNSNAQV----SADGKTVTA 167
L. crispatus LMG 12003 SlpnA   -VGFNFGPENKGKKITLVSSN--SKVTFGSDN-----------AKTVTV 172
L. crispatus MH315 LbsA        -VGFSFGSAMKNKTVTVKLAN--GELSGKNVTKN---------TDGSYKL 187
L. acidophilus ATCC 4356 SlpA -VSFNFGSENAGRTVTLGSAN--SNVKFTGTNSDNQT-ETNVS---TLKV 171
L. acidophilus ATCC 4356 SlpB GVSFNFGSTNANKTITLAFPKNVSAAGLADANKVSATSETSVDAGKTIQV 177
                                *.:.**.   .*.:*.   :  .                    :
```
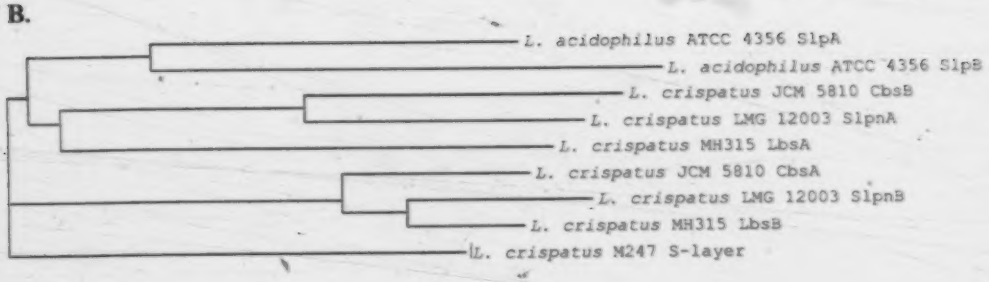

Fig. 1.7. Comparison of group A *acidophilus* S-protein sequences. **A**. ClustalW alignment. Blue boxes represent valine rich sites thought to be important for collagen and laminin binding and S-protein folding in CbsA [1]. Green boxes represent regions important for S-layer formation for SlpA [85]. Yellow boxes indicate surface exposed amino acids in SlpA [85]. Orange box indicates site important for collagen binding of CbsA [81]. "*" indicates identical amino acid in all sequences, ":" indicates very similar but different amino acids, "." indicates similar but different amino acids. Boxed amino acids indicates signal sequence. Arrow indicates start of SAC in SlpA. The GenBank accession numbers are as follows: ATCC 4356 SlpA, CAA61560; ATCC 4356 SlpB, CAA61561; JCM 5810 CbsA, AAB58734; JCM5810 CbsB, AAC28100; LMG 12003 SlpnA, AAF68971; LMG 12003 SlpnB, AAF68972; M247 S-layer, CAA07708; MH315 LbsA, BAC76686; MH315 LbsB, BAC76687. **B**. Phylogenetic tree based on protein sequence comparisons. **C**. Percent identity between protein sequences. **D**. Schematic of S-protein organization/functional domains based on data obtained from CbsA (top) and SlpA (bottom).

A.

```
L. crispatus LMG 12003 SlpnB   QLDKNGTVT--LNEVVLHATAKDFANPAVVNWYNTATNSVVSTGNIELFA 215
L. crispatus MH315 LbsB        KLDQNGTIT--LPEVVMNVTAKDFANPAVVNWYNTATNAVVSTGNIELFA 217
L. crispatus JCM 5810 CbsA     QLDKNGTTT--LPEVVMNVTAKNFANPTVVTWLNGTTSAPVTAGNITLYA 223
L. crispatus M247 S-layer      ELGQNGTLTTPISVQVSNVNALDLSNANGVNFYNASNGSQVTKGSVNVTA 225
L. crispatus JCM 5810 CbsB     TLDQNGTVS-GLTV-VERLVAYDATNTNDVVFYNIATGQPVNSGDAMVLA 215
L. crispatus LMG 12003 SlpnA   SLDQNGTAK-DLTVNISDVTAFNATNTNGVVFYNVTTGTQAHAGNAMVLA 221
L. crispatus MH315 LbsA        TLDQYGNAT-ELTY-TQSLKAYNQGNTNSVFFINQNSGTTETKGLYLTLA 235
L. acidophilus ATCC 4356 SlpA KLDQNGVAS-LTNVSIANVYAINTTDNSNVNFYDVTSGATVTNGAVSVNA 220
L. acidophilus ATCC 4356 SlpB KTDKNGVVS-FGSAQVLNVKVVETSDVRAVSFYDIQTGKTVENGTLSIVA 226
                                 .: *  .          . :  :   * : :  ..     *     *

L. crispatus LMG 12003 SlpnB   GSDAGKMNVAQVTSAALKKYHASNY--GTAANQE----------SSTISY 253
L. crispatus MH315 LbsB        GSDAGKMNVAQVVSATEKKYHASNY--GTKANQE----------SSTISY 255
L. crispatus JCM 5810 CbsA     GSDAGKMNVAQVVAEARKNYVAMGA--KVADF------------------ 253
L. crispatus M247 S-layer      G-LIGRLNVSTVASEILKNCAAYQVSNGKPVSQL----------PDQKAV 264
L. crispatus JCM 5810 CbsB     D-SNKQLNVAAILPAVKSNFTATQRVTVAQGNGNGTYSQDQINTVKINTT 264
L. crispatus LMG 12003 SlpnA   N-TQGQLNTAALLPAIESNYVAVQRVDSDSANGNGTYNFADFKHVNNIEF 270
L. crispatus MH315 LbsA        N-GNGELNVADVLANIEKQYTAVQYNDSKFMSSTEKDS-----PVTITTN 279
L. acidophilus ATCC 4356 SlpA D-NQGQVNVANVVAAINSKYFAAQY--ADKKLNTR----------TANT 256
L. acidophilus ATCC 4356 SlpB G-SNARANVQEIVNAFNAKYQASQLNNANSNANVRL----------TDNN 265
                               .    . *.  :      :  *

L. crispatus LMG 12003 SlpnB   SNNLVEALKAAGVEVKDN-WFVAPKSFTFNMTATANNNDASKTLAVTVSV 302
L. crispatus MH315 LbsB        TNNLKDALKAMNVDVDAQGWFVAPKSFTFNMTAKANNNDASSTLAVTVSV 305
L. crispatus JCM 5810 CbsA     TNNIKEALKAMNIDVDARGWFVAPKSFTFNLTAKSDVNDATATLPVTVNV 303
L. crispatus M247 S-layer      VADVNAALKAANIPVDNAGWFTAPISLSVNVKASSSINGVGCYFTCTVNV 314
L. crispatus JCM 5810 CbsB     TPEIKDQLEKAGIKIDANGNFTAPHSFKVTVKATSDVNGKSKELPVTFTV 314
L. crispatus LMG 12003 SlpnA   ATAIKDQLKAQNIDVGPQGFFKAPHTFTVKVKATSSINGKSEELPVTFTV 320
L. crispatus MH315 LbsA        KDAVIAELKKQNIPVNAAGNFTAPDTFTVTLNAKSSINGKTGQLVVTVSV 329
L. acidophilus ATCC 4356 SlpA EDAIKAALKDQKIDVNSVGYFKAPHTFTVNVKATSNTNGKSATLPVVVTV 306
L. acidophilus ATCC 4356 SlpB AQAVATMLRAQNIDVDAQGYFTAPASLSLTFHAESTQNNETAQLPVTVSV 315
                                  :   *.   : :     * ** ::.... * :  *.    :  ...*

L. crispatus LMG 12003 SlpnB   PN---GKDMTVPSQSKTIMHNAFYYDKN-GKRVGSDKVTRYNSATVAMNT 348
L. crispatus MH315 LbsB        PN---GKDMTVPSQSKTVMHNAFFYDKN-GKRVGSDKVTRYNSATVAMNT 351
L. crispatus JCM 5810 CbsA     PN---GKDTTVPSQSKTVMHNAYFYDKN-GKRVGSDKVTRYNSATVAMST 349
L. crispatus M247 S-layer      AN---GKDMTVPSQSKTIMHNAYYYDKD-AKRVGTDKLTRYNSVTVAMNT 360
L. crispatus JCM 5810 CbsB     AN---VAEPTVASQSKMIMHNAYYYKEDGTTRANNDKAKRYESVTVAMST 361
L. crispatus LMG 12003 SlpnA   AN---VADPVVPSQPKTIMHNAYYYKEDGTTRANNDKAKRYESVNVAMST 367
L. crispatus MH315 LbsA        PN---GKKTTVASQEKTIMHNAYYYDKD-AKRVGTDKVTRYNKVTVATST 375
L. acidophilus ATCC 4356 SlpA PN---VAEPTVASVSKRIMHNAYYYDKD-AKRVGTDSVKRYNSVSVLPNT 352
L. acidophilus ATCC 4356 SlpB TNGKEVTPSTVDSVSKSFMHNAYYYDKD-AKRVGTDSVKRYNSVSVLPNT 364
                               .*      ↑.* *  * .****::*.::  .*...*. .**:...*  .*

L. crispatus LMG 12003 SlpnB   TTINGKAYYEVIESGKATGKFINAANIDGTKRTLKHNAYVYKSSKKRANK 398
L. crispatus MH315 LbsB        TTINGKAYYEVIENGKATGKFINAANIDGTKRTLKHNAYVYKSSKKRANK 401
L. crispatus JCM 5810 CbsA     TTIKGKAYYEVIENGKATGKFINAANIDGTKRTLKHNAYVYKSSKKRANK 399
L. crispatus M247 S-layer      TTINGKAYYEVIENGKATGKFINADNIDGTKRTLKHNAYVYKTSKKRANK 410
L. crispatus JCM 5810 CbsB     KKIGDKNFYEVIKDGKATSMYINADNIDGTKRTLKHNAYVYKTSKKRANK 411
L. crispatus LMG 12003 SlpnA   KKIGNKDFYEVIKDGKATGMYINADNIDGTKRTLKHNAYVYKTSKKRANK 417
L. crispatus MH315 LbsA        TKIGDKTYYEVIENGKATGKYINADNIDGTKRTLKHNAYVYATSKKRANK 425
L. acidophilus ATCC 4356 SlpA TTINGKTYYQVVENGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANK 402
L. acidophilus ATCC 4356 SlpB TTINGKAYYQVVENGKAVDKYINAANIDGTKRTLKHNAYVYASSKKRANK 414
                               ..* .* :*:*::.***.. :*** ********************** :*******

L. crispatus LMG 12003 SlpnB   VVLKKGDTVVTYGGTYTFKNGKQYYKIYNNTEKTYVKASNF- 439
L. crispatus MH315 LbsB        VVLKKGTEVVTYGGAYTFKNGKQYYKIGNNTDKTYVKASNF- 442
L. crispatus JCM 5810 CbsA     VVLKKGTEVTTYGGAYTFKNGKQYYKIGNNTDKTYVKASNF- 440
L. crispatus M247 S-layer      VTLKKGTEVTTYGGTYTFKNGKQYYKIGNNTDKTYVKASNF- 451
L. crispatus JCM 5810 CbsB     VVLKKGEEVTTYGGTYTFKNGKQYYKIGNDTKKTYVKASNF- 452
L. crispatus LMG 12003 SlpnA   VVLKKGDTVVTYGGTYTFKNGKQYYKIYNNTEKTYVKASNF- 458
L. crispatus MH315 LbsA        FVLKKGEEVTTYGGTYTFKNGKQYYKIGNDTKKTYVKASNF- 466
L. acidophilus ATCC 4356 SlpA VVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDKTYVKVANFR 444
L. acidophilus ATCC 4356 SlpB VVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDKTYVKVANFR 456
                               ..****  *.***.:******::**** ::*.*****.:**
```

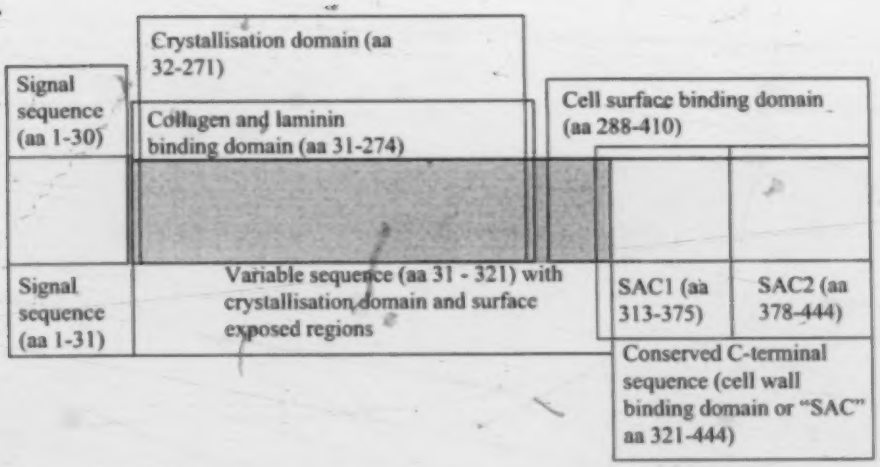
B.


L. acidophilus ATCC 4356 SlpA
L. acidophilus ATCC 4356 SlpB
L. crispatus JCM 5810 CbsB
L. crispatus LMG 12003 SlpnA
L. crispatus MH315 LbsA
L. crispatus JCM 5810 CbsA
L. crispatus LMG 12003 SlpnB
L. crispatus MH315 LbsB
L. crispatus M247 S-layer

C.

	L. acidophilus ATCC 4356 SlpA	*L. crispatus* LMG 12003 SlpnB	*L. crispatus* JCM 5810 CbsA	*L. crispatus* JCM 5810 CbsB	*L. crispatus* LMG 12003 SlpnA	*L. acidophilus* ATCC 4356 SlpB	*L. crispatus* M247 S-layer	*L. crispatus* MH315 LbsA	*L. crispatus* MH315 LbsB
L. acidophilus ATCC 4356 SlpA		57	48	49	52	43	55	50	48
L. acidophilus ATCC 4356 SlpB			45	40	40	43	45	45	46
L. crispatus JCM 5810 CbsA				46	48	76	53	48	84
L. crispatus JCM 5810 CbsB					71	45	47	49	46
L. crispatus LMG 12003 SlpnA						46	49	51	49
L. crispatus LMG 12003 SlpnB							50	46	85
L. crispatus M247 S-layer								52	52
L. crispatus MH315 LbsA									47
L. crispatus MH315 LbsB									

D.


Crystallisation domain (aa 32-271)
Signal sequence (aa 1-30)
Collagen and laminin binding domain (aa 31-274)
Cell surface binding domain (aa 288-410)
Signal sequence (aa 1-31)
Variable sequence (aa 31 - 321) with crystallisation domain and surface exposed regions
SAC1 (aa 313-375)
SAC2 (aa 378-444)
Conserved C-terminal sequence (cell wall binding domain or "SAC" aa 321-444)

presence of surface exposed regions and crucial structural regions (near amino acids 30, 67, 88, and 156 of the mature protein) involved in crystal formation. Cleavage of SlpA with trypsin and chymotrypsin revealed that potential cleavage sites on the edges of SAN and in the middle region (*ca.* 150 amino acids) are accessible to protease, whereas other internal potential cleavage sites were not cut. Insertions of *c-myc* at amino acids 7, 45, and 125 formed in-tact S-layer crystals, which could be expressed in *L. acidophilus* ATCC 4356. Due to the presence of a functional WT *slp*A gene in the chromosome, the researchers found that a maximum of 5% of the total SlpA protein expressed contained the insertion. However, combined PCR, fluorescence microscopy and FACS analysis suggested that a subset of the transformed *L. acidophilus* underwent a double cross-over event whereby the mutant *slp*A protein was inserted into the genome and only mutant S-protein was present on the cell surface. Variable regions were able to accept up to 19 amino acids without affecting S-layer crystallization, and this development holds potential for the production of surface-exposed epitopes by lactobacilli to be used for expression of epitopes on the surface of live vaccine preparations (refer to the discussion of this in section 1.3.2.5, below). The data regarding the domains of SlpA are summarized in Fig. 1.7D.

Both S-layer protein genes from two strains of *L. crispatus* (JCM 5810 and LMG 12003) were expressed in *E. coli* and His-tag purified by Sillanpää et al. [81]. As with SlpA from *L. acidophilus* ATCC 4356, the N-terminal end of CbsA was found to be essential for S-layer formation, while the C-terminal end played no role in S-layer formation. It was also observed that His-CbsA (from *E. coli*) bound to *L. crispatus* JCM 5810 cells, and formed large aggregates of S-protein as observed by light microscopy.

When *L. crispatus* JCM 5810 cells that had been stripped of their S-layers were added to the His-CbsA aggregates, the cells were embedded in the aggregates. Purified CbsA (from *L. crispatus*) and His-CbsA (from *E. coli*) were found to bind radiolabelled, soluble collagens-I and -IV, however other *L. crispatus* S-layer proteins (CbsB, SlpnA, SlpnB) studied in the same fashion did not. Further, the binding ability of the purified S-proteins to soluble collagens was determined to be similar to that obtained for the *L. crispatus* cells themselves [95]. Hybrid S-proteins made from CbsA and SlpnB and SlpA were made [81], and their solubility and immobilized collagen binding ability was determined. It was found that aa 1 to 287 of His-CbsA was necessary for high affinity binding to collagen-I and -IV. A much weaker collagen-I binding site was detected in aa 288-401 of His-CbsA. Deletion studies and production of short peptide fragments from CbsA were also used to further investigate the binding of CbsA to collagens. In particular, mutations at amino acids 257, 258 and 260 had a strong effect in reducing collagen-I and -IV binding. It was found that mutated S-proteins that failed to form supramolecular crystals also failed to bind collagens, therefore suggesting a functional relationship between the formation of crystalline S-layers and affinity for collagen. Interestingly, the investigators analysed isolates from all other *L. acidophilus* groups for collagen binding and cross-reaction to the *cbs*A probe (-6 to +813, corresponding to aa –2 to +271 of the mature protein). The only isolate that reacted with the probe was another *L. crispatus* isolate, however five isolates studied bound to collagens. Unfortunately, they did not state which species they studied, nor which ones bound collagens. They noted that CbsA and SlpnB, although highly homologous (Fig. 1.7B & C), have varied collagen binding activities.

To further analyse the collagen binding ability of CbsA, frozen sections of chicken colon (a collagen rich site) were exposed to *L. crispatus* JCM 5810 with in-tact and stripped S-layers. When the S-layer was in-tact, the strain bound to connective tissue (apical and basolateral) of the chicken colon, but not epithelial cells [81]. However, when stripped of its S-layer, *L. crispatus* JCM 5810 failed to bind chicken tissue.

In a further study [62], *cbs*A was cloned and expressed in *L. casei*. The authors found that the S-protein did not attach to the *L. casei* cell surface, but could be localized there by engineering a *cbs*A::*prt*P (proteinase P) fusion using the C-terminal anchor of *prt*P. CbsA epitopes were found to be present in the *L. casei* cell surface, although the authors expressed a concern that the folding of the proteins may be different in *L. casei* and that there may be fewer S-proteins on the surface of *L. casei* as compared to *L. crispatus*. The collagen binding ability of the surface anchored CbsA in *L. casei* was lower than that for wild type *L. crispatus*, likely due to one or both of these factors.

Antikainen et al. [1] studied CbsA from *L. crispatus* in a similar approach as that used by Smit et al. [84-86]. Fragments of the *cbs*A gene were cloned and expressed in *E. coli* and the collagen binding ability of the polymer formed by these truncated CbsA fragments was analysed [1]. The valine rich sites of aa 30-32 and 269-273 (Fig. 1.7A) appeared to be responsible for S-layer polymerization, and mutations of these regions resulted in alterations in the polymer structure as seen in TEM. Fragments comprised of amino acids 288-410 adhered to *L. crispatus* cells stripped of their S-layer and to teichoic acids extracted from *L. crispatus* JCM 5810. Segments of *cbs*A fused to the gene encoding a cell wall anchor were cloned and expressed in *L. casei*, [1,62]. Fragments including aa 31-278 adhered to insoluble laminin and collagen-IV and frozen chicken

colon sections but fragments without the valine rich sites (above) failed to adhere [1]. Thus, as with SlpA from *L. acidophilus*, the N-terminus of CbsA is responsible for crystal formation and the C-terminus is responsible for attachment to the cell wall, although the presence of the C-terminal domain was required for the formation of a sheet-like S-layer morphology. Further, the N-terminus is responsible for adherence to tissue, specifically collagens and laminins in the chicken colon. The data regarding the domains of CbsA are summarized in Fig. 1.7D.

Taken together, the information published regarding CbsA and SlpA suggest that the N-terminal domains of these proteins contain the signal sequence, crystallisation domains, and surface exposed regions which are likely involved in environmental interactions such as adherence to host tissue components (Fig. 1.7D). The C-terminus of these S-proteins contains the cell wall binding domain and is responsible for anchoring the S-proteins to teichoic acids in the cell wall (Fig. 1.7D). Alignments of CbsA, SlpA, and other S-protein sequences (Fig. 1.7A) indicate high conservation in the N-terminal signal sequence and the C-terminal anchoring domain, and regions of limited homology between these two conserved domains (i.e. the "variable region"). A large amount of data implicate the S-layer in adherence to tissue components and to human cell lines and *ex vivo* chicken intestines [1,39,41,62,81,95], although the exact role of S-layers in adherence *in vivo* has not been examined. An important limitation to these studies is that the removal of the S-layer with LiCl or GnCl may also remove minor cell surface proteins, which may also play a role in adherence to tissues and/or aggregation. Therefore, the data provided through genetic engineering of S-proteins or their domains (e.g. [1,3,41,62,81]) presents a stronger case for the importance of S-layers in adherence.

For example, Takahashi et al. [90] observed that the whole extracellular S-protein extract from *L. acidophilus* JCM 1034 had a higher ability to adhere to human colonic mucous than the purified S-protein itself, suggesting the presence of other adherence components in the extracellular S-layer. Lorca et al. [60] found other cell surface proteins to be involved in binding of *L. acidophilus* CRL 639 to collagen-I and fibronectin. In another study, cell surface glycoproteins in *L. acidophilus* JCM 1132 were found to be responsible for adherence to chicken intestinal lectin [69]. Greene and Klaenhammer [36] observed adherence of *L. acidophilus* strains [BG2FO4 and NCFM/N2] to Caco-2 cells that was not mediated by the S-layer. Indeed, the relationship of S-proteins and other extracellular proteins on the surface of lactobacilli is not well understood. While adherence to collagens and laminin has been demonstrated *in vitro*, it remains to be determined 1) if S-proteins adhere to these tissue components in the conditions experienced *in vivo* and 2) if these tissue components are exposed and available for adherence *in vivo*. Further, in organs such as the crop, collagens and laminins are unlikely to be exposed and therefore adherence of crop lactobacilli could occur via a different mechanism. The first paper demonstrating the *in vivo* effects of the spontaneous mutation of an unidentified persistence factor was recently published [17]. An isogenic spontaneous *L. crispatus* M247 mutant does not bind mucous, does not aggregate and does not persist when fed to humans (i.e. was not detected in faeces or biopsies), although the correlation between the phenotype and potential genetic differences (i.e. deletion or alteration of a key gene, such as the S-protein gene) have not been published [17].

Further, the information published by Boot et al. [12] contains very important implications for the expression of S-proteins by lactobacilli. Phase variation in

Campylobacter fetus S-proteins resulting in host immune system avoidance was elucidated by Dworkin and Blaser [23,24], and the discovery of a similar mechanism in *L. acidophilus* has implications for the effects of lactobacilli in the gastrointestinal tract and other highly selective environments [13]. It seems possible that *L. acidophilus* and other lactobacilli with S-layers express different S-proteins when in the gastrointestinal tract and when grown in the laboratory or in other environments (such as when growing on plant sources or in industrial situations, i.e. yogurt) [99]. No work has yet been performed to determine potential phase variation of *Lactobacillus* S-layers outside of the laboratory environment. However, it should be noted that expression of EPS outside of the S-layer may be responsible for altered *in vitro* effects. Schneitz et al. [79] found that strains of *L. acidophilus* isolated from the gastrointestinal tract of chickens subcultured in the laboratory for several generations showed a different colony morphology, decreased aggregation in broth culture, decreased adherence to chicken intestinal epithelial cells, and decreased competitive exclusion of *Salmonella in vivo*. TEM of these cultures indicated the presence of an additional layer outside the S-layer, which may have been EPS. Further, Lorca et al. [60] found decreased fibronectin and collagen-I binding of a strain of *L. acidophilus* during stationary phase when EPS was observed.

1.3.2.4. S-proteins from other lactobacilli

The S-proteins from other lactobacilli have been characterized and are summarized in Table 1.3. *L. brevis* has been shown to carry and express S-protein genes. *L. brevis* ATCC 8287 was found to contain only one S-protein gene [46,102]. *L. brevis* ATCC 14869 carries three genes, two of which were differentially expressed *in vitro* in the absence or presence of oxygen and may affect the colony morphology [43]. However,

unlike the situation in *L. acidophilus* ATCC 4356, *L. brevis* ATCC 14869 does not appear to induce expression of a previously silent gene through chromosomal recombination involving the promoters, as shown by real time PCR amplification of the *slp*D promoter region. Reporter analysis indicated that S-protein variation in *L. brevis* ATCC 14869 is likely controlled by an unidentified soluble factor. The N-terminus of *L. brevis* S-proteins is likely responsible for adherence to cell lines and tissue components such a fibronectin [41].

L. helveticus has been shown to contain one S-protein gene by Boot et al. [11]. The first S-protein in *L. helveticus* was detected in strain ATCC 12046 by Lortal et al. in 1992 [61], followed by the sequencing of the *slp*H gene from *L. helveticus* CNRZ 892 [16]. Unlike the S-proteins in the *L. acidophilus* group, the entire protein sequences of *L. helveticus* S-proteins show high homology (data not shown). No information regarding the regulation of the S-protein gene in *L. helveticus* has been published, nor information regarding adherence of *L. helveticus* strains to tissue components or human or animal cell lines. The C-terminal domain of all S-proteins sequenced from *L. helveticus* strains show high homology to the C-terminus of the group A *acidophilus* S-proteins (data not shown), therefore suggesting a similar function.

1.3.2.5. Applications of S-layers of lactobacilli

The 16S rDNA sequences of lactobacilli have been used to identify *Lactobacillus* spp. [56], however, as Ventura et al [100] note, there are few differences between these sequences in homofermentative, S-layer producing species (Fig. 1.2) such as *L. acidophilus*, *L. crispatus*, *L. gallinarum*, and *L. helveticus*. Therefore, attempts to use genes that exhibit more variability to rapidly identify *Lactobacillus* spp. have been

reported. S-layers have been used as a taxonomic tool to identify *Lactobacillus* spp. Horie et al. [40] designed primers to regions within the S-layer genes conserved among *L. crispatus* isolates. When a high fidelity polymerase was used, these primers were able to amplify DNA from only *L. crispatus* and not *L. acidophilus*, *L. gallinarum*, and *L. amylovorus*. Ventura et al. [100] used a primer set and probe (a fragment of the PCR product produced by the primers) specific for the *L. helveticus* S-layer gene to differentiate previously studied *L. helveticus* isolates from other lactobacilli.

S-layers can be removed from bacterial cells, and have been found to spontaneously re-crystallise upon removal of the compounds used for their extraction from the cell [82]. This property makes them tremendously useful for a variety of biotechnological uses such as ultrafiltration membranes or immunoassays. S-layers have been used for the stabilization of macroscopic preparations of biological lipid membranes, which can be used for a number of technologies such as diagnostics, electronic devices, and drug delivery systems. Genetic manipulation of S-layer protein genes could also be used for variety of unique biotechnological developments, including live oral vaccines. LAB such as lactobacilli are an attractive candidate for oral vaccine vectors as they have a history of safe ingestion and are naturally found in the gastrointestinal microflora [65]. Further, LAB have been found to have adjuvant properties, which may be due to S-layers [82]. SlpA from *L. acidophilus* ATCC 4356 [85] and *L. brevis* ATCC 8287 [2] are able to accept foreign DNA and display it on the cell surface, which could be useful for oral vaccines. *L. brevis* ATCC 8287 *slp*A was successfully expressed on a low copy number vector in different hosts (*Lc. lactis* and *L.*

plantarum) [47]. However, this application of LAB requires significant research and testing before its acceptance in routine prevention of human and animal illness.

1.4. Thesis objectives

Since Fuller's detailed analysis of the crop microflora in the 1970s, little work has been done to further elucidate the bacterial populations present in this organ and determine their possible interactions with the host. Further, the development of novel molecular techniques to analyse bacterial populations with greater specificity (and in some instances, without the need to culture the organisms), has made it possible to investigate the crop microflora in greater detail than has been previously possible. Further, given the taxonomic reorganizations of the *Lactobacillus* genus, the true identity of the members of the gastrointestinal microflora of the chicken can now be elucidated using molecular techniques. Therefore, we performed an initial ecological analysis using culture-independent techniques as well as isolation and speciation of lactobacilli to determine the members of the crop microflora of broilers raised under commercial conditions (Chapter 2). To develop a better understanding of the potential colonization and persistence factors of these lactobacilli, the S-proteins of one species of the *L. acidophilus* group that was frequently isolated from the chicken crop were analysed (Chapter 3).

1.5. References

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Chapter 2: Detection and Identification of *Lactobacillus* Species in the Crop of Broilers of Different Ages Using PCR-Denaturing Gradient Gel Electrophoresis and Amplified Ribosomal DNA Restriction Analysis

2.1. Introduction*

The digestive tract of mammals and avians is home to a diverse collection of bacterial species, collectively referred to as the gut microflora [33]. The microflora is known, from gnotobiotic animal studies, to influence the biochemistry, immunology, physiology, and non-specific resistance to intestinal infection of the host [11]. The impact of the gut microflora on the nutritional status of farm animals is of particular interest, especially where intensive farming practices are used [6].

The crop, ileum, caeca, and colon of poultry are known to harbour bacterial populations [21,32]. Recent reports have investigated the composition of the ileal [17] and caecal [42] microflora, analysed using bacteriological culture and culture-independent methods. Lactobacilli are numerous in the ileum of broilers, whereas the caecal microflora is dominated by obligately anaerobic and yet-to-be-cultivated bacteria. From the results of culture-based studies, the microflora of the crop has a simple composition and is dominated by lactobacilli [21,32]. Colonization of the surface of the stratified, squamous epithelium of the crop by lactobacilli has been reported by Fuller [8]

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* This chapter is based on the paper "Detection and Identification of *Lactobacillus* Species in the Crop of Broilers of Different Ages Using PCR-Denaturing Gradient Gel Electrophoresis and Amplified Ribosomal DNA Restriction Analysis" by Le Luo Guan, Karen E. Hagen, Gerald W. Tannock, Doug R. Korver, Gaylene M. Fasenko, and Gwen E. Allison, published in Applied and Environmental Microbiology (2003, Volume 69, issue 11 p. 6750-6757).

and by Morishita et al. [24]. *Lactobacillus salivarius*, *Lactobacillus fermentum/Lactobacillus reuteri*, and *Lactobacillus acidophilus* were the species most commonly detected [21,32]. These studies were conducted prior to the re-classification of *L. acidophilus*, which has been divided into two DNA homology groups containing six related species [7,14,19] (Chapter 1 section 1.1.2). Even with molecular methods, it is difficult to distinguish between members of this group (Chapter 1 Fig. 1.2). Methods that have been successfully used include: DNA-DNA hybridization and various biochemical properties [7,14,19]; analysis of whole cell protein profiles by SDS-PAGE [5,10,16,27] randomly amplified polymorphic DNA (RAPD) [5,10,16,28]; sequencing of 16S and 23S rDNA and elongation factor Tu [4,18,35,36]; oligonucleotide probes and primers for species-specific hybridizations and PCR, respectively [27-29,39]; ribotyping [30]; amplified fragment length polymorphism (AFLP) [10]; and amplified ribosomal DNA restriction analysis (ARDRA) [23,29,38]. There is a clear need, however, to develop simple methods for the differentiation of members of the *L. acidophilus* group to ensure large scale microbial ecological studies are logistically possible.

A detailed analysis of the crop microflora of broilers using nucleic acid-based methods has not previously been reported. Our study therefore had two main aims. Firstly, we used PCR-DGGE to compare the crop microflora of birds of different ages. Secondly, we cultured lactobacilli from the crop of the birds and used an ARDRA technique by which the members of the *L. acidophilus* group could be rapidly identified.

The results that we have obtained indicate that there is variation in the composition of the crop microflora between birds and between age groups. Since the crop microflora acts as a bacterial inoculum of the digesta that passes through the remainder of the gut

[9], knowledge of the composition of this bacterial collection is critical in understanding the contribution of the microflora members to the well-being of the avian host. The base-line information that we have obtained will be essential in planning husbandry methods that utilize feed supplements other than antimicrobial drugs for the efficient production of broilers.

2.2. Materials and methods

2.2.1. Animals, treatment and sampling.

Ross 308 broiler chicks (Aviagen Inc. Huntsville, AL), originating from the Teamstra farm (flock 29), were obtained from a commercial hatchery (Lilydale Hatchery, Edmonton, AB, Canada) and raised at the Alberta Poultry Research Centre (APRC), University of Alberta by the ARPC staff under the supervision of Doug Korver and Gaylene Fasenko. Chicks (n=125) were placed in each of 8 floor pens with fresh straw as the litter material. Stocking density in each of the floor pens was 609 cm^2/bird. At each sampling time, one or two birds from each pen were selected randomly and in such a way that the stocking density was maintained. Birds were raised under conditions similar to commercial broiler production, with feed and water provided *ad libitum*. The wheat-based diet was provided as a starter from 0 to 21 days of age, as a grower from 22 to 35 days of age, and as a finisher from 36 to 42 days of age (Table 2.1). In addition to slight differences in antimicrobial concentrations, all three diets contained the same components although the ratio of corn, wheat, canola oil, soy and amino acids were modified in order to meet the nutritional needs of the developing birds (Table 2.1). All nutrients were included at levels to meet or exceed the National Research Council's [25] recommendations for broiler chickens.

Table 2.1. Dietary nutrient and ingredient composition for chicken sampled

Ingredient	Starter	Grower	Finisher
	g/1000 g diet		
Wheat	645.5	644.7	680.1
Soybean Meal	210.5	205.5	167.0
Corn Gluten Meal	32.0	5.5	N/A[8]
Meat Meal	50.0	50.0	50.0
Salt	3.70	3.70	3.70
Ground Limestone	10.00	9.75	9.95
Dicalcium Phosphate	8.90	8.20	7.45
L-Lysine HCl	2.090	1.335	1.330
L-Threonine	0.69	0.84	0.77
D,L-Methionine	1.42	1.39	1.19
Canola Oil	19.20	53.10	62.99
BMD[1]	0.50	0.5	0.5
Monensin[2]	0.50	0.5	N/A
Choline Chloride[3]	5.0	5.0	5.0
Vitamin E[4]	3.0	3.0	3.0
Broiler Premix[5]	5.0	5.0	5.0
Pellet Binder[6]	1.5	1.5	1.5
Avizyme 1302[7]	0.50	0.5	0.5
Calculated Nutrient Composition			
AME_N, Kcal/kg	2,906	3,086	3,181
Crude Protein	22.2	20.2	18.6
Arginine	1.30	1.23	1.12
Lysine	1.20	1.10	1.00
Methionine	0.52	0.47	0.43
Methionine + Cystine	0.90	0.82	0.76
Tryptophan	0.23	0.22	0.19
Ca	0.95	0.92	0.90
Available P	0.42	0.40	0.38
Sodium	0.18	0.18	0.18

[1]Bacitracin Methylene Disalicylate, Alpharma Canada Inc. Mississauga, Ontario, Canada L5N 1W1

[2]Monensin Sodium, Elanco Animal Health, Guelph, Ontario, Canada N1G 4T2

[3]Choline chloride premix provided 100 mg per Kg of diet

[4]Vitamin E premix provided 15 IU per kg of diet.

[5]Broiler Premix provided the following per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,500 IU; vitamin E 35 IU; menadione, 2 mg; D-pantothenic acid, 14 mg; riboflavin, 5 mg; folic acid, 0.8 mg; niacin, 65 mg; thiamine, 2 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.015 mg; biotin, 0.18 mg; iodine, 0.5 mg; iron, 100 mg; zinc, 80 mg; manganese, 70 mg; copper, 8.5 mg; selenium, 0.1 mg.

[6]Lignoplex Plus

[7]Danisco Animal Nutrition, Marlborough, Wiltshire, SN8 1XN, United Kingdom

[8]N/A - Not applicable

Birds from two experiments were used in this work (refer to section 2.3.3, Results). The first experiment (Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee [FAPWC] protocol number 2001-43B) started on October 20, 2001 and lasted until November 1, 2001. The 1st experiment was a pilot study designed to establish standard operating procedures for the dissection and sampling techniques, therefore, the number and ages of birds, and the sections sampled differed for experiment 1 and 2. In the first experiment, 2 to 3 chickens of 2, 5, 8, 11, and 14 days of age were sampled. For each sample time, the crop and/or caeca from two chickens were analysed.

The 2nd experiment (FAPWC 2002-12B) started on April 19, 2002 and ended on May 31, 2002. In the 2nd experiment, 86 birds were sampled as follows: 6 birds on day 0 (day of hatch) and 10 each on 1, 3, 7, 14, 21, 35, and 42 days of age. Additional day 0 samples were obtained from 10 birds raised in identical conditions on August 6, 2002. For each bird, the crop was analysed and the rest of the gastrointestinal tract was frozen as described below.

For both experiments, chickens were euthanised at the APRC following standard protocols for cervical dislocation (FAPWC SOP P-06). Chicken carcasses were placed in a plastic or Styrofoam cooler and transported to the University of Alberta campus, with an estimated travel time of 20 min. Upon arrival at the Agriculture/Forestry Centre (Ag/For), the cooler was filled with ice. Carcasses were left on ice until they were dissected. Dissections were conducted in the food microbiology laboratory (Ag/For 2-50) or the animal dissection room (Ag/For 1-51). After dissection, carcasses were placed in the animal carcass disposal storage (Ag/For 3-60) for incineration.

Sterilized stainless steel dissection equipment was used to dissect the birds. When switching between different areas and organs of the gastrointestinal tract, dissection equipment was changed or immersed in 70% ethanol, flamed, and cooled. When obtaining samples for analysis, all manipulations were conducted on sterile Petri dishes. The dissection was commenced by cutting the skin of the neck and subsequently tearing the skin down the length of the bird to expose the underlying tissue and muscle. The crop was removed first, the abdominal cavity was exposed by cutting through the breast muscle and bone, and the caeca and the intestine were removed.

For both experiments, crop samples were obtained by taking a section parallel to and between the entry and exit of the oesophagus into the crop. The section of crop tissue and corresponding contents weighing approximately 1 g, or the entire crop if weighing less than 1 g, were transferred into a sterile 15 mL Pyrex Tenbroeck tissue grinder (Corning, Acton, MA; Cat. no. 7727-15) and 9 mL of 0.85% saline was added. The glass pestle was moved and rotated such that homogenization of the tissue was obtained (in some circumstances small particles of tissue fat remained solid). The crop homogenate was then poured into a sterile 15 mL BlueMax Jr polypropylene Falcon tube (Becton Dickinson, Franklin Lakes, NJ; Cat. no. 352097). For each bird in both experiments, a portion of the crop homogenate was used immediately for selective enumeration and collection of lactobacilli (below). For the 2nd experiment, 1 mL of crop homogenate was stored at -80°C for nucleic-acid based analysis of the bacterial communities in a 2 mL sterile conical screw cap tube with O-ring (Axygen, Union City, CA; Cat. no. SCT200CS).

For the 1st experiment, caecal samples were obtained by taking a cross section of

the caecal pouch dissected approximately 1/3-1/2 the length from the ileocaecal junction. The caecal samples were weighed and homogenized as outlined for the crop, and used immediately for selective enumeration and collection of lactobacilli (below). For future analysis of the gastrointestinal *Lactobacillus* population, intestinal and caecal samples were collected in the 2nd experiment. The entire small intestine was placed onto a large Petri dish and sectioned into the duodenum, jejunum and ileum, which were frozen in separate 15 mL Falcon tubes. Both lobes of the caeca were placed in 15 mL BlueMax Jr polypropylene Falcon tube or 50 mL BlueMax Falcon tube (BD Cat. no. 352070). All samples were stored at -80°C.

For instances where tissue and contents were separated, the section of the crop or caecum was opened and washed once in sterile 0.85% saline. The tissue was then homogenized and plated (below) and the wash was also used for selective enumeration.

2.2.2. Propagation and enumeration of lactobacilli.

The tissue homogenates were used to make a series of ten-fold dilutions (10^{-2} to 10^{-7}) in sterile 0.85% NaCl. For each dilution, 100 µl was spread plated on agar resulting in a final dilution of 10^{-3} to 10^{-8}. For the first experiment, LBS agar (*Lactobacillus* selective (BBL), Becton Dickinson, Cockeysville, MD; Cat. no. 211327, adjusted to pH 5.5 with glacial acetic acid as per manufacturers instructions) containing 20% clarified tomato juice (obtained by centrifugation of tomato juice at 8000xg [LBS+TJ]). For the second experiment, LBS agar, acidified as outlined above, was used. The plates were incubated at 37°C for 48 hr in an anaerobic environment (Thermo Forma anaerobic system with 5% CO_2, 10% H_2, balance N_2). The CFU of presumptive lactobacilli per gram of crop for each bird was determined from the number of colonies on countable (i.e.

those containing 20-200 colonies) LBS plates. A total of 10 colonies per bird were selected for further characterization as follows. The colony morphologies on the countable LBS plates were recorded and the prevalent colony morphology noted. Each unique colony type was selected, although one colony type was often selected more than once as the plates rarely contained 10 or more different colony morphology types. Often similar colonies of differing sizes were observed and these were selected for analysis rather than using colonies that were similar in both morphology and size. The selected colonies were picked and streaked heavily on MRS (deMann, Rogosa, and Sharpe (Difco), Becton Dickinson, Sparks, MD; Cat. no. 0881-08) agar plates using a sterile plastic loop and incubated for 36-48 hrs under anaerobic conditions as outlined above. The bacterial growth was scraped off the plate with a sterile plastic loop and suspended in labelled, sterile MRS broth containing 50% v/v glycerol in 2 mL sterile conical screw cap tube with O-ring. Cultures were stored at -80°C. For routine propagation of *Lactobacillus* reference strains and crop isolates (Table 2.2), MRS glycerol stocks were streaked onto MRS agar and incubated at 37°C under anaerobic conditions as outlined above.

2.2.3. Standard molecular biology techniques

Polymerase chain reaction (PCR) was always performed in a laminar flow cabinet. All PCR reagents, tips, and tubes were sterilized prior to use by autoclaving for 15 min at 121°C at 24 psi. With each PCR reaction, a negative control containing 1 µL of sterile MilliQ H_2O was used in replacement of the template DNA. PCR reagents and template DNA were stored at -20°C. PCR deoxynucleotide triphosphate (dNTP) mix was made from Invitrogen (Burlington, ON) Cat. no. 10297-018, oligonucleotides were synthesized by Invitrogen and Recombinant Taq (*Thermus aquaticus*) Polymerase

Table 2.2. Reference strains used

Strain	Relevant Characteristics
Lactobacillus acidophilus ATCC[1] 4356	Type strain
Lactobacillus amylovorus ATCC 33620	Type strain
Lactobacillus aviarius subsp. *aviarius* ATCC 43234	Type strain
Lactobacillus crispatus ATCC 33820	Type strain
Lactobacillus fermentum ATCC 14931	Type strain
Lactobacillus gallinarum ATCC 33199	Type strain
Lactobacillus gasseri ATCC 33323	Type strain
Lactobacillus johnsonii ATCC 33200	Type strain
Lactobacillus johnsonii ATCC 11506	
Lactobacillus reuteri ATCC 23272	Type strain
Lactobacillus salivarius subsp. *salivarius* ATCC 11741	Type strain

[1]ATCC - American Type Culture Collection

(Invitrogen Cat. no. 10342-020) was used. PCR reactions were set up on ice, vortexed gently to mix, centrifuged briefly and placed into a pre-warmed GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA; Cat. no. 4134879).

For genomic DNA extraction, filter sterilized or autoclaved reagents were used where possible (sodium dodecyl sulphate [SDS] and NaOH solutions were not sterilized). Unless otherwise stated, solutions were sterilized by autoclaving. Sterile plastic tubes and baked glassware were used when needed. Agarose used for DNA analysis was purchased from Invitrogen (Cat. no. 15510-027). Agarose and DGGE gels were stained with 1 μg/mL and 5 μg/mL ethidium bromide (EtBr, Bio-Rad, Hercules, CA; Cat. no. 161-0433), respectively. Acrylamide for denaturing gradient gel electrophoresis (DGGE) was purchased from Bio-Rad (Cat. no. 161-0148). Images were captured with an Alphadigidoc system (AlphaInnotech, San Leandro, CA; Cat. no. AD-1201-1) using a UV-transilluminator for agarose and DGGE gels.

Alignments for 16S rDNA sequences were performed with ClustalW using the MegAlign module of the Lasergene software package (DNAStar Inc, Madison, WI).

2.2.4. DNA extraction from crop homogenates and crop isolates.

Bacterial DNA from the crop homogenate was extracted by Leluo Guan as described by Walter et al. [41]. Briefly, the frozen crop homogenate was allowed to thaw on ice and was then centrifuged at 14,600xg for 5 min at 4°C. The pellet was washed twice with 1 mL of TN150 buffer (10 mM Tris-HCl; 0.15M NaCl, pH 8.0) After resuspending the pellet in the same, the cells were lysed by physical disruption with zirconium-silica beads (0.1 mm diameter, BioSpec, Bartlesville, OK; Cat. No, 11079101z) in a BioSpec Mini Bead-Beater-8 at 4800 rpm for 3 minutes. Three phenol-

chloroform-isoamyl alcohol (25:24:1) extractions were performed on each sample using TE-saturated phenol (Invitrogen Cat. no. 15513-047) and the DNA was precipitated with cold ethanol and dissolved in 30 µl of 1X TE buffer (10 mM Tris-HCl, 1mM EDTA [ethylene diamine tetra acetic acid] [pH 8.0]). The presence of DNA was checked using a 0.7% agarose gel prior to PCR.

DNA was extracted from *Lactobacillus* type strains and crop isolates by Karen Hagen and Leluo Guan as described by Walter et al. [40]. All centrifugation for DNA extraction was performed in a refrigerated microcentrifuge at 4°C at 14600xg for 3 min. Cultures were heavily streaked on MRS agar and incubate for 48 hrs, then scraped into a sterile 1.7 mL microcentrifuge tube containing 1 mL sterile MilliQ H_2O and the cells were centrifuged. The cells were then washed once in 1 mL TN150 buffer and resuspended in the same. The suspension was then transferred into a 2 mL sterile conical screw cap tube with O-ring with 0.3 g of 0.1 mm zirconium beads and lysed as above. The samples were cooled on ice for 30-60 min. An aliquot of 500 µL was removed into a sterile 1.7 mL microcentrifuge tube and extracted with the same volume of phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated as outlined above. The DNA precipitate was dissolved in 500 µl of TE buffer and treated with 0.1 mg/mL RNAse A for at least 1 hour at 37°C and then extracted with phenol:chloroform:isoamyl alcohol and the ethanol precipitation was repeated. The purified DNA was then resuspended in 20 µL 1X TE buffer. For working solutions, the DNA was diluted 1/20 dilutions in 1X TE buffer, and the stocks and dilutions were stored at -20°C.

2.2.5. PCR-DGGE analysis of crop DNA with universal bacterial primers and primers specific for lactic acid bacteria

PCR was conducted using either individual or pooled crop DNA as template. The pooled samples were prepared by combining the crop DNA (1 µl of each) from all 10 crops collected at the same sampling time. The V2-V3 region of the 16S rRNA gene was amplified from the crop DNA using universal bacterial primers HDA1-GC and HDA2 (HDA-PCR), following the protocol of Walter et al. [40]. The V3 region of the 16S rDNA was amplified from the total crop DNA using group specific bacterial primers Lac1 and Lac2-GC (Lac-PCR) [41]. The HDA and Lac PCR products were subjected to DGGE by Karen Hagen and Leluo Guan (8% acrylamide gel with a gradient of 20-55% urea and formamide [HDA PCR-DGGE] or 30-45% urea and formamide [Lac PCR-DGGE]) using the Bio-Rad DCode Universal Mutation Detection System at 130 V for 4 hrs in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 60°C as outlined by Walter et al. [40,41]. Identification ladders for DGGE were prepared by combining the HDA or Lac PCR products prepared from DNA extracted from the type and reference *Lactobacillus* strains (Table 2.2). DGGE gels were stained with EtBr for 15-20 min, then destained in MilliQ H_2O for 30 min and viewed by UV transillumination. DGGE profiles were compared using Dice's similarity coefficient (D_{sc}) with the Bionumerics software package (Applied Maths, Austin, TX). When performing D_{sc} analysis, profiles were only compared within the same gel, not between gels. Average D_{sc} was calculated by adding the values of single profile comparisons for the age range stated and dividing by the total number of D_{sc} values.

2.2.6. Identification of bacteria by sequencing DNA fragments.

DNA fragments generated by Lac PCR were extracted from DGGE gels by Leluo

Guan as described by Knarreborg et al. [17]. Following purification, the DNA was re-amplified with the Lac1 and Lac2 (without GC clamp) primers using the PCR protocol described above. The resulting PCR products were purified with the QIAquick purification kit (Qiagen, Mississauga ON; Cat. no. 20021), ligated into pGEM-T (Promega, Madison, WI, Cat. no. A1360), and used to transform *Escherichia coli* JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-,m_k+), *rel*A1, *sup*E44, Δ(lac-proAB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15] [22]. Transformants were plated on LB agar [31] containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), and IPTG (isopropyl-beta-D-thiogalactopyranoside) at concentrations of 100 μg/mL, 80 μg/mL and 0.5 mM, respectively. Several colonies were subcultured and the plasmid DNA was isolated following the alkaline lysis procedure described by Sambrook et al. [31]. Plasmid DNA was used as template in a PCR reaction with Lac1 and Lac2-GC primers, and the PCR products were analyzed by DGGE in order to compare the migration of the cloned DNA with the migration of the desired band from the original PCR-DGGE crop profile. The pGEM-T insert DNA was amplified using T7 and SP6 primers, and sequencing was conducted by the Agricultural, Food and Nutritional Science Biotech Core, University of Alberta. The sequences were compared with those in the GenBank database using the BLAST (basic local alignment search tool) algorithm [1].

2.2.7. Identification of *Lactobacillus* isolates with ARDRA

The total DNA extracted from each reference strain or crop isolate was used as template for PCR amplification of the 16S rRNA gene plus the entire 16S-23S rRNA intergenic region (16-23S rDNA). The following primers were used to amplify the 16-23S rDNA (2 kb): Lb16a ($^{5'}$GTGCCTAATACATGCAAGTCG$^{3'}$) corresponding to nt 36-

57 of the 16S rDNA of *Lactobacillus crispatus* ATCC 33820 (GenBank accession no. LCR17362) (this study, Fig. 2.1, designed by Karen Hagen) and 23-1B ($^{5'}$GGGTTCCCCCATTCGGA$^{3'}$) corresponding to nt 123-113 of *Lactobacillus* 23S rDNA, which was developed by Tannock et al. [35]. PCR reactions were performed using the following program: 94°C for 5 min; 25 cycles of 94°C for 45 sec, 53°C for 30 sec, and 72°C for 1 min 30 sec; and concluded with a final extension at 72°C for 7 min. Each PCR reaction contained 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 1 pmol/μL each oligonucleotide and 0.025 U/μL Taq Polymerase and 1 μL dilute (1/20) template DNA. The PCR products were subjected to restriction digestion using *Hae*III (Invitrogen Cat. no. 15205-016, 10U in REact 2 buffer with 50 mM Tris-HCl [pH8.0], 10 mM $MgCl_2$, 50 mM NaCl) or *Mse*I (5U, New England Biolabs [Pickering, ON] Cat. no. R0525L in 1X NEBuffer 2 with 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT [dithiothreitol] pH 7.9) at 37°C for 1-2 hrs. One tenth a volume of 3 M NaOAc (pH 5.2-5.6) was added to the restriction digest and it was ethanol precipitated as outlined above (section 2.2.4). The tubes were centrifuged at 14600xg for 20 min at 4°C. The pellets were dried and resuspended in 10 μL 1X TE buffer, mixed with 2 μL 6X DNA loading dye (30% glycerol, 0.25% Bromophenol Blue) and run on 2% agarose gel in 1X TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA).

To confirm the species designation of the crop isolates, the V2-V3 region of the 16S rDNA was sequenced using primers Lb16a, HDA-2, Lac1 and Lac2 (without GC clamp). Sequencing and analysis were conducted as above by Leluo Guan.

L. acidophilus ATCC 4356 ----------
L. amylovorus ATCC 33620 ----------
L. crispatus ATCC 33820 ----------
L. gallinarum ATCC 33199 ----------
Lb16a ----------
L. gasseri ATCC 33323 CAAGCAATAAATTTGAGATAACTCAAAGAAAGTTTTAGAGCTAAACGATA 50
L. johnsonii ATCC 33200 ----------
L. acidophilus ATCC 4356 ----------NNAAAACGAGAGTTTGATCCTGGCTCAGGAC 31
L. amylovorus ATCC 33620 ----------NNNTANAATGAGAGTTTGATCCTGGCTCAGGAC 33
L. crispatus ATCC 33820 ----------AGAGTTTGATNNTGGCTCAGGAC 23
L. gallinarum ATCC 33199 ----------CTGGCTCAGGAC 12
Lb16a ----------
L. gasseri ATCC 33323 AAAAGCTCATTTTCAAGAAGGAAAATGAGAGTTTGATCCTGGCTCAGGAC 100
L. johnsonii ATCC 33200 ----------
L. acidophilus ATCC 4356 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAAC 81
L. amylovorus ATCC 33620 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAAC 83
L. crispatus ATCC 33820 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACTAAC 73
L. gallinarum ATCC 33199 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCAGAACCAGC 62
Lb16a ----------GTGCCTAATACATGCAAGTCG---------- 21
L. gasseri ATCC 33323 GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGA 150
L. johnsonii ATCC 33200 -------GGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGA 43
****
L. acidophilus ATCC 4356 AGATTC----ACTTCGGT--GATGACGTTGGGNAA-CGCTAGCGGCGGAT 124
L. amylovorus ATCC 33620 AGATTT----ACTTCGGT--AATGACGTTGNAAA----CNAGCGGCGGAT 123
L. crispatus ATCC 33820 AGATTT----ACTTCGGT--AATGACGTTAGGAA--AGCGAGCGGCGGAT 115
L. gallinarum ATCC 33199 AGATTT----ACTTCGGT--AATGACGCTGGGGA--CGCGAGCGGCGGAT 104
Lb16a ----------
L. gasseri ATCC 33323 TGAATTTGGTGCTTGCACCAGATGAAACTAGATACAAGCGAGCGGCGGAC 200
L. johnsonii ATCC 33200 TGATTTTAGTGCTTGCACTAAATGAAACTAGATACAAGCGAGCGGCGGAC 93

Fig. 2.1: Alignment of partial *L. acidophilus* 16S rDNA from type strains with primer Lb16a using ClustalW. "*" indicates identical nucleotide in all sequences. The GenBank accession numbers are as follows: ATCC 4356, LBARR16SA; ATCC 33620, LBARR16SD; ATCC 33820, LCR17362; ATCC 33199, LGA417737; ATCC 33323, LGA242968; ATCC 33200, LBARR16SAZ. Entire 16S rDNA alignment of all species belonging to the *L. acidophilus* group is presented in Fig. 1.2.

2.3. Results

2.3.1. PCR-DGGE profiles of the crop microflora.

To investigate the total bacterial community in the crop throughout the production period, individual and pooled crop DNA were used as template in HDA-PCR followed by DGGE analysis. First, the PCR-DGGE profiles of ten individual birds from each sampling time were compared on a single gel. Three to four representative profiles for each sampling time were then selected and run on the same gel for comparative purposes (Fig. 2.2). Comparison of the PCR-DGGE profiles between individual birds revealed that there was diversity in the composition of the bacterial community among birds of the same age. Major and minor fragments (as detected by intensity of staining) were noted in each bird's profile, but after day 3 the major fragments co-migrated with those in the *Lactobacillus* identification ladder (Fig. 2.2). All of the day 1 profiles had similar major fragments that were not present in the profiles of older birds (Fig. 2.2).

For comparison of crop microflora among birds of different ages, the individual crop DNA were pooled for each sampling time and PCR-amplified using the HDA primers. The corresponding DGGE profiles were analysed on the same gel (Fig. 2.3) and the similarity of the profiles was compared using D_{sc} (Table 2.3). This analysis revealed that the composition of the microflora changed markedly between day 1 and day 7 and then remained relatively stable from day 14 to day 42. The major bands in day 3 to day 42 profiles co-migrate with those of the *Lactobacillus* identification latter (Fig. 2.3). Comparison of migrations of the HDA PCR-DGGE fragments of *Lactobacillus* reference strains showed that *L. acidophilus*, *L. amylovorus*, *L. crispatus* and *L. gallinarum* fragments had almost identical migration distances (Fig. 2.4A) and could not be easily

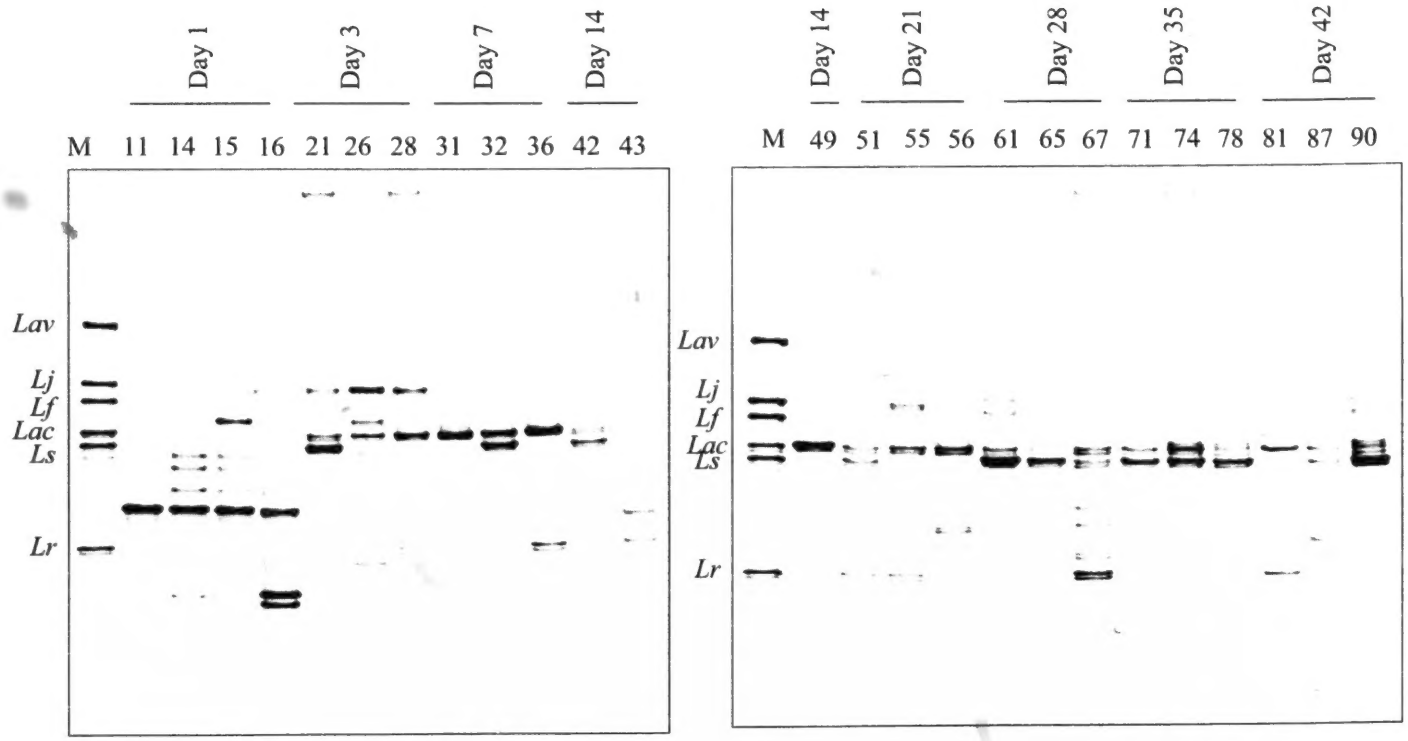

Fig. 2.2. PCR-DGGE profiles (22 to 55% denaturing gradient gel) generated from individual crop DNA from day 1 through day 42 chickens, using primer pairs HDA1-GC and HDA2. Numbers above lanes indicate individual chicken numbers. Identification ladder (M) is composed of PCR products from the following reference strains of *Lactobacillus*: *Lav, L. aviarius* ATCC 43234; *Lj, L. johnsonii* ATCC 33200; *Lf, L. fermentum* ATCC 14931; *Lac, L. acidophilus* ATCC 4356; *Ls, L. salivarius* ATCC 11741; and *Lr, L. reuteri* ATCC 23272 (Table 2.2).

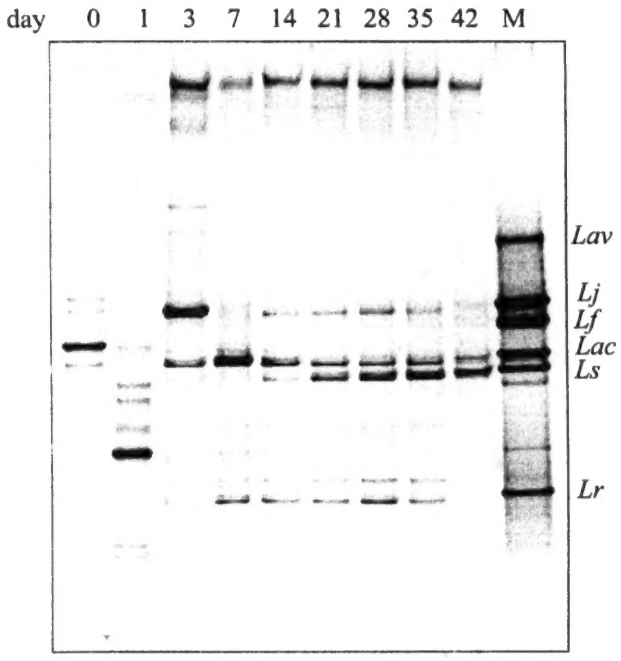

Fig. 2.3. PCR-DGGE profiles (22 to 55% denaturing gradient gel) generated from pooled crop DNA, using primer pair HDA1-GC and HDA2. The sample time is indicated at the top of the gel. Identification ladder (M) composed of PCR products from the following reference strains of *Lactobacillus*: *Lav, L. aviarius* ATCC 43234; *Lj, L. johnsonii* ATCC 33200; *Lf, L. fermentum* ATCC 14931; *Lac, L. acidophilus* ATCC 4356; *Ls, L. salivarius* ATCC 11741; and *Lr, L. reuteri* ATCC 23272 (Table 2.2).

Table 2.3. Comparison of DGGE profiles generated from HDA and Lac PCR using Dice's similarity coefficient (D_{sc}).

Sample time (day)	Sample time (day)								
	0[1,3]	**1**[1,2,3]	**3**	**7**	**14**	**21**	**28**	**35**	**42**
0	100	66.7	44.5	16.7	33.3	33.3	36.4	36.4	25
1	12.5	100	80	46.2	61.5	61.5	66.7	66.7	44.5
3	26.1	24	100	61.5	76.9	76.9	66.7	66.7	44.5
7	11.8	31.6	61.5	100	87.5	75	66.7	66.7	33.3
14	30	36.4	69	78.3	100	87.5	80	80	50
21	33.3	30	59.3	76.2	91.7	100	93.3	93.3	66.7
28	28.6	26.1	66.7	66.7	88	88	100	100	72.7
35	21.1	19.1	57.2	63.6	80	84.6	84.6	100	72.7
42	30	27.3	48.3	52.2	69.2	66.7	74.1	64	100

[1]Grey fill - percent similarity of HDA PCR-DGGE profiles indicated
[2]No fill - percent similarity of Lac PCR-DGGE profiles indicated
[3]Black fill - identity for both HDA and Lac PCR-DGGE

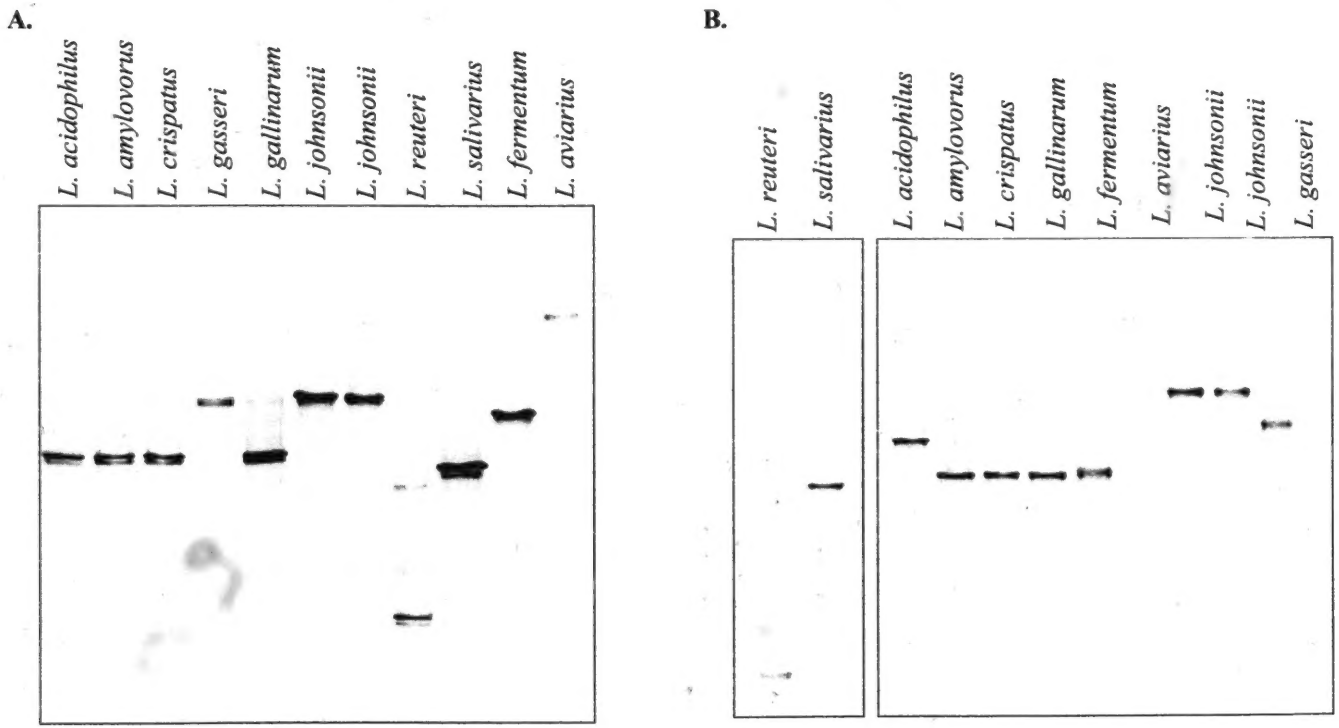

Fig. 2.4. PCR-DGGE profiles generated from *Lactobacillus* type strains used in this study. (Table 2.2). **A.** HDA-DGGE profiles on a 22 to 55% denaturing gradient gel.**B.** Lac-DGGE profiles on a 30 to 45% denaturing gradient gel. The corresponding species are indicated at the top of the gels.

differentiated by DGGE. HDA PCR-DGGE, however, could distinguish between group A and B *L. acidophilus*.

2.3.2. PCR-DGGE profiles of broiler crop Lactic Acid Bacteria (LAB) microflora.

To further investigate the LAB component of the crop microflora, group-specific PCR-DGGE was conducted using Lac1 and Lac2-GC primers on both individual (Fig. 2.5) and pooled crop DNA (Fig. 2.6). Similar to the analysis of individual birds with HDA PCR-DGGE, the composition of the LAB microflora varied from bird to bird, with most crop profiles containing a fragment that co-migrated with the *L. crispatus* type strain (Fig. 2.5) in 85.7% (60/70) of the profiles after day 1 (data not shown). *L. johnsonii* was also well represented, as it was detected in 84.3% (59/70) of the profiles after day 1 (data not shown). Fragments generated from *L. crispatus, L. gallinarum, L. amylovorus* and *L. fermentum* DNA migrated to the same position in Lac PCR-DGGE gels (Fig. 2.4B). These species could not therefore be differentiated by Lac PCR-DGGE. As with HDA PCR-DGGE, the fragments in the group-specific profiles co-migrated with those of the *Lactobacillus* identification ladder from day 3 onwards (Fig. 2.5).

Comparison of the LAB microflora in the crop over time revealed similar trends to those obtained with the HDA PCR-DGGE (Fig. 2.6, Table 2.3). The DGGE profiles from the pooled crop DNA showed that the composition of the LAB population remained relatively stable from day 14 to day 42 (average D_{sc} value among these pooled profiles was 80%, Table 2.3). While HDA PCR-DGGE profiles for days 0 and 1 were very different from the others, Lac PCR-DGGE profiles for these time points showed less disparity.

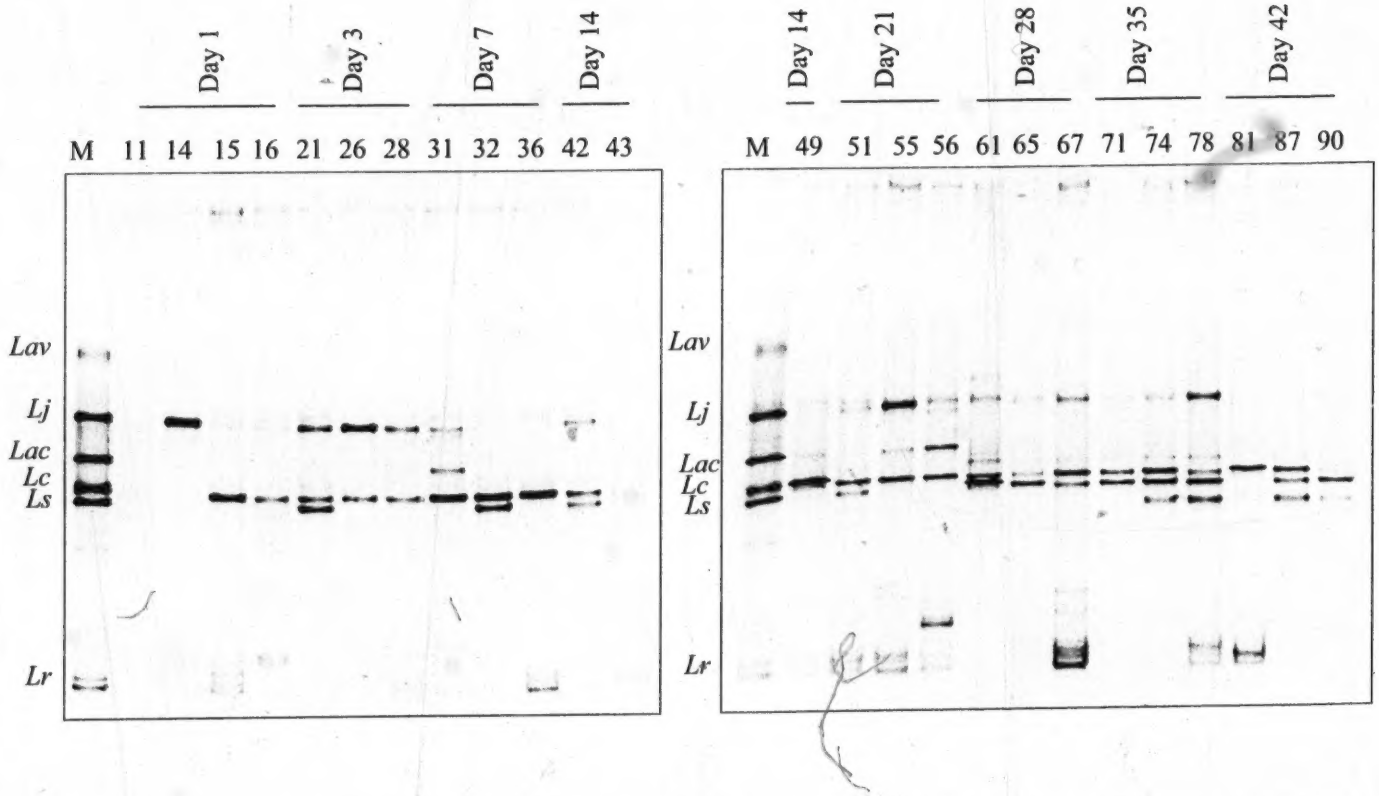

Fig. 2.5. PCR-DGGE profiles (30 to 45% denaturing gradient gel) generated from individual crop DNA from day 0 through day 42 chickens, using primer pairs Lac1 and Lac2-GC. Numbers above lanes indicate chicken number Identification ladder (M) is composed of PCR products from the following reference strains of *Lactobacillus*: *Lav, L. aviarius* ATCC 43234; *Lj, L. johnsonii* ATCC 33200; *Lac, L. acidophilus* ATCC 4356; *Lc, L. crispatus* ATCC 33820; *Ls, L. salivarius* ATCC 11741; and *Lr, L. reuteri* ATCC 23272 (Table 2.2).

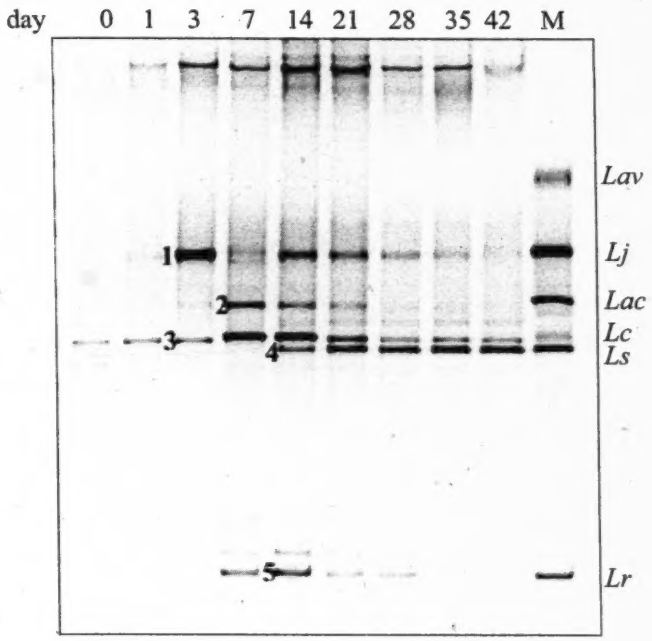

Fig. 2.6. PCR-DGGE profiles (30 to 45% denaturing gradient gel) generated from pooled crop DNA, using primer pair Lac1 and Lac2-GC. The sample time is indicated at the top of the gel. Identification ladder (M) composed of PCR products from the following reference strains of *Lactobacillus*: *Lav, L. aviarius* ATCC 43234; *Lj, L. johnsonii* ATCC 33200; *Lac, L. acidophilus* ATCC 4356; *Lc, L. crispatus* ATCC 33820; *Ls, L. salivarius* ATCC 11741; and *Lr, L. reuteri* ATCC 23272 (Table 2.2). Numbered fragments were extracted and sequenced as outlined in the text and Table 2.4.

To confirm the species designations for the major fragments observed for days 3 to 14, DNA fragments were extracted, cloned and sequenced (Table 2.4). The sequences obtained for fragments 1, 2, 3, 4 and 5 (Fig. 2.6) confirmed the presence of *L. johnsonii*, *L. acidophilus*, *L. crispatus/L. gallinarum*, *L. salivarius* and *L. reuteri*, respectively (Table 2.4). Similar to profiles from individual birds, all pooled profiles contained *L. crispatus/L. gallinarum/L. amylovorus* and/or *L. fermentum* (Fig. 2.5). In general, *L. salivarius* was present from day 14 to 42; *L. acidophilus* was present from day 7 to 21; *L. johnsonii* was present from day 3 to 35; and *L. reuteri* was present at all ages (Fig. 2.6).

2.3.3. Enumeration of lactobacilli from chicken crops.

For each sampling time, the CFU of presumptive lactobacilli per gram of crop for each bird was determined and used to calculate the mean log CFU/g and standard deviation (Tables 2.5 & 2.6) and the comprehensive data for the 2nd experiment are summarized in Table 2.7. For day 1 birds from the 2nd experiment, countable plates were only obtained for five out of ten crops, giving a large standard deviation. From day 1 to day 7, the number of lactobacilli increased 1000-fold, reaching a maximum population (10^9 CFU/g) at day 7 (Table 2.7, Appendix Fig. A.1). From day 14 onwards, the numbers of lactobacilli stabilized at ~10^8 CFU/g, corresponding to the period of compositional stability revealed by the Lac PCR-DGGE results.

Table 2.4. Summary of the sequence analysis of the 340 bp V2-V3 region of 16S rDNA fragments eluted from the Lac PCR-DGGE gel (Fig. 2.6).

Fragment no.	Species	Accession no.	Identity
1	*L. johnsonii*	AJ002515	100.0%
2	*L. acidophilus*	M58802	99.0%
3	*L. crispatus*	AF257097	99.4%
	L. gallinarum	AJ417737	99.4%
4	*L. salivarius subsp. salivarius*	AF335475	99.4%
5	*L. reuteri*	AF 257097	98.7%

Table 2.5. Summary of chicken samples collected and used for isolation of lactobacilli in the 1st experiment

Chicken no.	Age of chicken (days)	Weight of chicken (g)	Type of crop sample taken	Weight of crop (g)	CFU on LBS+TJ	CFU/g in crop[2]	Type of caecal sample taken	Weight of caeca (g)	CFU in caeca on LBS+TJ	CFU/g in caeca
1	2	52.95	Whole crop	1.67	1.18E+09	7.07E+08	Both	0.32	1.01E+09	3.15E+09
3	2	51.4	Whole crop	0.62	3.66E+07	5.90E+07	Both	0.33	3.50E+08	1.06E+09
1457	5	83.56	Whole crop	2.794	ND[1]	ND	Both	0.559	ND	ND
			Section with contents	1.11	3.81E+08	3.43E+08	ND	ND	ND	ND
			Section tissue only	0.143	1.47E+07	1.03E+08	ND	ND	ND	ND
1450	5	77.32	Whole crop	3.185	ND	ND	Both	1.510	ND	ND
			Section with contents	0.601	3.90E+09	6.49E+09	Section with contents	0.165	8.50E+08	5.15E+09
1460	5	96.43	Whole crop	1.904	ND	ND	Both	2.147	ND	ND
			Section with contents	0.506	1.05E+09	2.08E+09	Section with contents	0.403	6.30E+08	1.56E+09
1453	8	113.6	Whole crop	3.299	ND	ND	Both	0.483	ND	ND
			Section with contents	1.074	3.90E+08	3.63E+08	Section with contents	0.168	6.30E+07	3.75E+08

[1]ND - Not done

[2]Grey fill – CFU/g in crop and caeca from samples that isolates were obtained from

Table 2.5. Continued

Chicken no.	Age of chicken (days)	Weight of chicken (g)	Type of crop sample taken	Weight of crop (g)	CFU on LBS+TJ	CFU/g in crop[2]	Type of caecal sample taken	Weight of caeca (g)	CFU in caeca on LBS+TJ	CFU/g in caeca
1436	8	89.27	Whole crop	2.702	ND	ND	Both	0.56	ND	ND
			Section with contents	0.45	3.00E+08	6.67E+08	Section with contents	0.073	1.485E+07	2.03E+08
1444	8	129.43	Whole crop	1.769	ND	ND	Both	0.885	ND	ND
			Section with contents	0.435	1.67E+08	3.84E+08	Section with contents	ND	1.90E+07	ND
1461	11	146.92	Whole crop	4.551	ND	ND	One	0.977	ND	ND
			Section with contents	1.016	7.60E+08	7.48E+08	Section with contents	0.134	2.01E+08	1.50E+09
1458	11	228.64	Whole crop	1.563	ND	ND	One	0.896	ND	ND
			Section with contents	0.362	6.40E+06	1.77E+07	Section with contents	0.169	3.20E+06	1.89E+07

[1]ND - Not done

[2]Grey fill – CFU/g in crop and caeca from samples that isolates were obtained from

Table 2.5. Continued

Chicken no.	Age of chicken (days)	Weight of chicken (g)	Type of crop sample taken	Weight of crop (g)	CFU on LBS+TJ	CFU/g in crop[2]	Type of caecal sample taken	Weight of caeca (g)	CFU in caeca on LBS+TJ	CFU/g in caeca
1441	14	301.74	Whole crop	2.67	ND	ND	One	2.027	ND	ND
			Section with contents	0.461	3.60E+08	7.81E+08	Section with contents	0.429	1.43E+08	3.33E+08
1464	14	247.24	Whole crop	2.08	ND	ND	Both	3.636	ND	ND
			Crop section	0.49	ND	ND	Section with contents	2.07	ND	ND
			Crop section tissue only	0.259	3.20E+07	1.24E+08	Caecal section, tissue only	0.062	1.26E+06	2.03E+07
			Crop contents from above section	0.231	1.90E+08	8.23E+08	Caecal contents from above section	2.008	1.90E+08	9.46E+07

[1]ND - Not done

[2]Grey fill – CFU/g in crop and caeca from samples that isolates were obtained from

Table 2.6. Summary of chicken samples collected and used for isolation of lactobacilli in 2nd experiment

Sample no.	Chicken no.	Weight of chicken (g)	Age (days)	Weight of whole crop (g)	Weight of crop section used (g)	CFU on LBS	CFU/g in crop
1	N/A[1]	36.37	0	0.23	0.23	<1.0E+02	<1.0E+02
2	N/A	39.26	0	0.26	0.26	<1.0E+02	<1.0E+02
3	N/A	35.44	0	0.22	0.22	<1.0E+02	<1.0E+02
4	N/A	36.37	0	0.20	0.20	<1.0E+02	<1.0E+02
5	N/A	40.94	0	0.24	0.24	<1.0E+02	<1.0E+02
1A	N/A	36.15	0	0.21	0.21	<1.0E+02	<1.0E+02
11	2322	50.86	1	2.50	1.15	6.2E+04	5.39E+04
12	2378	42.00	1	2.14	0.88	2.8E+04	3.18E+04
13	2454	41.24	1	0.30	0.30	<1.0E+02	<1.0E+02
14	2527	41.88	1	2.10	0.80	5.6E+06	7.00E+06
15	2319	41.70	1	0.62	0.62	1.16E+07	1.87E+07
16	2334	44.11	1	2.70	0.79	5.5E+05	6.96E+05
17	4349	40.70	1	1.70	1.00	<1.0E+02	<1.0E+02
18	2352	32.60	1	0.45	0.45	<1.0E+02	<1.0E+02
19	2354	50.80	1	2.31	0.85	<1.0E+02	<1.0E+02
20	4340	40.90	1	0.56	0.56	<1.0E+02	<1.0E+02
21	2618	59.10	3	2.38	0.85	3.66E+08	4.30E+08
22	2432	56.20	3	1.94	0.89	1.16E+09	1.30E+09
23	2590	54.04	3	1.42	0.91	3.78E+08	4.15E+08
24	2420	66.40	3	2.42	1.09	1.49E+08	1.37E+08
25	4315	51.17	3	2.70	0.90	1.82E+08	2.02E+08
26	4319	63.45	3	0.60	0.60	2.07E+07	3.45E+07
27	2312	69.10	3	1.27	0.80	7.8E+08	9.75E+08
28	2370	60.50	3	0.90	0.90	4.1E+08	4.56E+08
29	2342	69.90	3	3.00	1.16	2.83E+09	2.44E+09
30	2524	57.75	3	0.21	1.00	1.41E+09	1.41E+09
31	4307	94.20	7	2.04	0.97	1.29E+09	1.33E+09
32	4331	72.27	7	0.70	0.70	4.50E+08	6.43E+08
33	2300	90.40	7	1.24	0.90	5.10E+08	5.67E+08
34	2610	96.40	7	1.35	0.85	2.50E+08	2.94E+08
35	2429	103.30	7	1.80	0.95	8.50E+08	8.95E+08
36	2452	94.73	7	2.24	1.10	3.90E+09	3.55E+09
37	4327	88.12	7	2.80	1.20	2.40E+09	2.00E+09
38	4304	89.90	7	0.94	0.94	8.40E+08	8.94E+08
39	2431	112.6	7	0.92	0.92	2.60E+08	2.83E+08
40	2291	102.9	7	3.05	0.95	4.10E+09	4.32E+09
41	2387	211.07	14	1.10	1.10	1.59E+08	1.45E+08
42	2566	229.11	14	2.12	1.25	1.86E+08	1.49E+08

[1]N/A – Not applicable

[2]No Lb – No lactobacilli cultivated

Table 2.6. Continued

Sample no.	Chicken no.	Weight of chicken (g)	Age (days)	Weight of whole crop (g)	Weight of crop section used (g)	CFU on LBS	CFU/g in crop
43	2415	233.20	14	2.70	1.30	2.20E+07	1.69E+07
44	4308	155	14	4.36	1.26	1.17E+09	9.29E+08
45	2612	229.50	14	0.88	0.88	2.20E+07	2.50E+07
46	2280	237	14	1.80	1.30	6.30E+06	4.85E+06
47	2326	198.5	14	5.70	1.21	2.90E+09	2.40E+09
48	2343	208.00	14	1.40	1.02	3.50E+08	3.43E+08
49	4291	161.20	14	2.10	1.09	1.80E+09	1.65E+09
50	2299	211.18	14	0.60	0.60	2.60E+07	4.33E+07
51	2331	415	21	2.76	1.06	1.30E+08	1.23E+08
52	4334	370	21	7.78	1.20	3.50E+08	2.92E+08
53	2511	252	21	3.64	1.19	3.30E+08	2.77E+08
54	2404	464	21	2.71	1.18	1.34E+08	1.14E+08
55	2619	395	21	13.05	1.28	5.80E+08	4.53E+08
56	2409	417	21	13.2	1.33	1.48E+09	1.11E+09
57	2309	631	21	9.05	1.70	2.50E+08	1.47E+08
58	2353	304	21	4.64	1.38	8.90E+08	6.45E+08
59	4335	338	21	2.41	1.04	4.50E+08	4.33E+08
60	2412	373	21	6.98	0.98	5.40E+08	5.51E+08
61	2411	636	28	2.47	1.03	1.34E+08	1.30E+08
62	2316	667	28	6.42	1.53	5.40E+08	3.53E+08
63	2365	1005	28	6.01	1.54	2.05E+08	1.33E+08
64	4301	1083	28	15.18	1.80	2.25E+09	1.25E+09
65	2383	886	28	3.96	1.01	5.10E+07	5.05E+07
66	2376	816	28	16.12	2.08	1.42E+09	6.83E+08
67	2323	787	28	5.03	1.26	5.90E+08	4.68E+08
68	2408	845	28	2.73	1.02	2.35E+08	2.30E+08
69	N/A	609	28	9.30	1.27	1.46E+09	1.15E+09
70	4326	795	28	6.61	1.6	3.40E+08	2.13E+08
71	2298	1308	35	3.98	0.99	9.00E+07	9.09E+07
72	2407	1566	35	13.71	2.96	8.10E+08	2.74E+08
73	2509	1216	35	12.44	2.06	3.80E+08	1.84E+08
74	4328	1425	35	4.39	1.26	1.56E+08	1.23E+08
75	2348	909	35	2.25	1.41	1.20E+08	8.51E+07
76	no tag, pen 14	1360	35	23.52	2.19	4.70E+08	2.15E+08
77	2428	1460	35	21.72	2.54	1.51E+09	5.94E+08
78	2307	1100	35	14.49	2.25	8.40E+08	3.73E+08
79	4436	1103	35	6.71	1.68	6.30E+08	3.75E+08
80	2546	1012	35	12.86	1.39	1.50E+07	1.08E+07
81	4362	1479	42	51.6	2.78	2.02E+09	7.25E+08

[1]N/A – Not applicable

[2]No Lb – No lactobacilli cultivated

Table 2.6. Continued

Sample no.	Chicken no.	Weight of chicken (g)	Age (days)	Weight of whole crop (g)	Weight of crop section used (g)	CFU on LBS	CFU/g in crop
82	2448	2088	42	21.97	1.74	6.60E+07	3.79E+07
83	2371	1701	42	5.75	1.25	2.50E+08	2.00E+08
84	2425	1984	42	16.87	2.55	1.17E+09	4.59E+08
85	2340	2082	42	21.28	3.06	4.50E+07	1.47E+07
86	2426	1623	42	8.50	2.03	8.10E+08	3.99E+08
87	4357	2027	42	9.82	1.87	1.79E+08	9.57E+07
88	2296	1955	42	20.00	3.54	1.60E+09	4.51E+08
89	4294	1677	42	24.78	3.35	2.56E+09	7.64E+08
90	2481	1618	42	13.78	3.31	3.80E+08	1.15E+08
91	4212	42.00	0	0.37	0.37	No Lb[2]	No Lb
92	2647	39.70	0	0.43	0.43	No Lb	No Lb
93	3310	39.30	0	0.30	0.30	No Lb	No Lb
94	2538	35.40	0	0.24	0.24	No Lb	No Lb
95	2634	41.00	0	0.47	0.47	No Lb	No Lb
96	2592	41.10	0	0.28	0.28	No Lb	No Lb
97	4142	40.40	0	0.39	0.39	No Lb	No Lb
98	4232	40.80	0	0.38	0.38	No Lb	No Lb
99	2757	42.80	0	0.37	0.37	No Lb	No Lb
100	4216	45.40	0	0.45	0.45	No Lb	No Lb

[1]N/A – Not applicable

[2]No Lb – No lactobacilli cultivated

Table 2.7. Average CFU presumptive lactobacilli per gram of crop isolated from broilers of various ages in the 2nd experiment.

Age (Days)	Average log CFU lactobacilli/g of crop homogenate
0	<2
1	5.84±1.23
3	8.65±0.55
7	9.00±0.41
14	8.14±0.90
21	8.51±0.33
28	8.48±0.45
35	8.20±0.49
42	8.27±0.57

2.3.4. Identification of lactobacilli and differentiation between the *L. acidophilus* group isolates using ARDRA.

The Lac PCR-DGGE analysis indicated that most crops contained *L. reuteri*, *L. salivarius* and representatives of different species of the *L. acidophilus* group. In order to rapidly and accurately identify the *Lactobacillus* isolates obtained from each bird, an ARDRA technique was developed. Initially, only the 16S rDNA gene was amplified and digested from the type strains, however species specific patterns were not obtained for members of the *L. acidophilus* group (data not shown). Tannock et al. [35] used the sequence of the 16S-23S rRNA intergenic region to speciate various members of the *L. acidophilus* group, and *in silico* restriction analysis of the intergenic region supported inclusion of this region in order to generate species-specific patterns. The 16-23S rDNA (2 kb) was amplified with primers Lb16a and 23-1B and PCR products generated from type strains were digested with *Hae*III. Identical banding patterns were observed for *L. amylovorus* ATCC 33620 and *L. gallinarum* ATCC 33199. The banding pattern for *L. acidophilus* ATCC 4356 was similar to that obtained for ATCC 33620 and ATCC 33199, but differed slightly in the number and size of fragments below 100 bp (Fig. 2.7). The banding pattern for *L. crispatus* ATCC 33820 differed from that of ATCC 33620, ATCC 33199, and ATCC 4356 in that the second largest fragment was ~ 350 bp instead of ~ 400 bp (Fig. 2.7) and this difference was used to discriminate *L. crispatus* from the other group A species. The *Hae*III banding pattern for *L. johnsoni* ATCC 33200 and ATCC 11506, and *L. gasseri* ATCC 33323 were the same, but were different from *L. crispatus* and the other group A species. Unique fragmentation patterns were also evident for *L. reuteri* ATCC 43272, *L. salivarius* subsp. *salivarius* ATCC 11741, *L. fermentum* ATCC 14931 and *L. aviarius* ATCC 43234 (Fig. 2.7). Subsequently, the *Hae*III-ARDRA of the

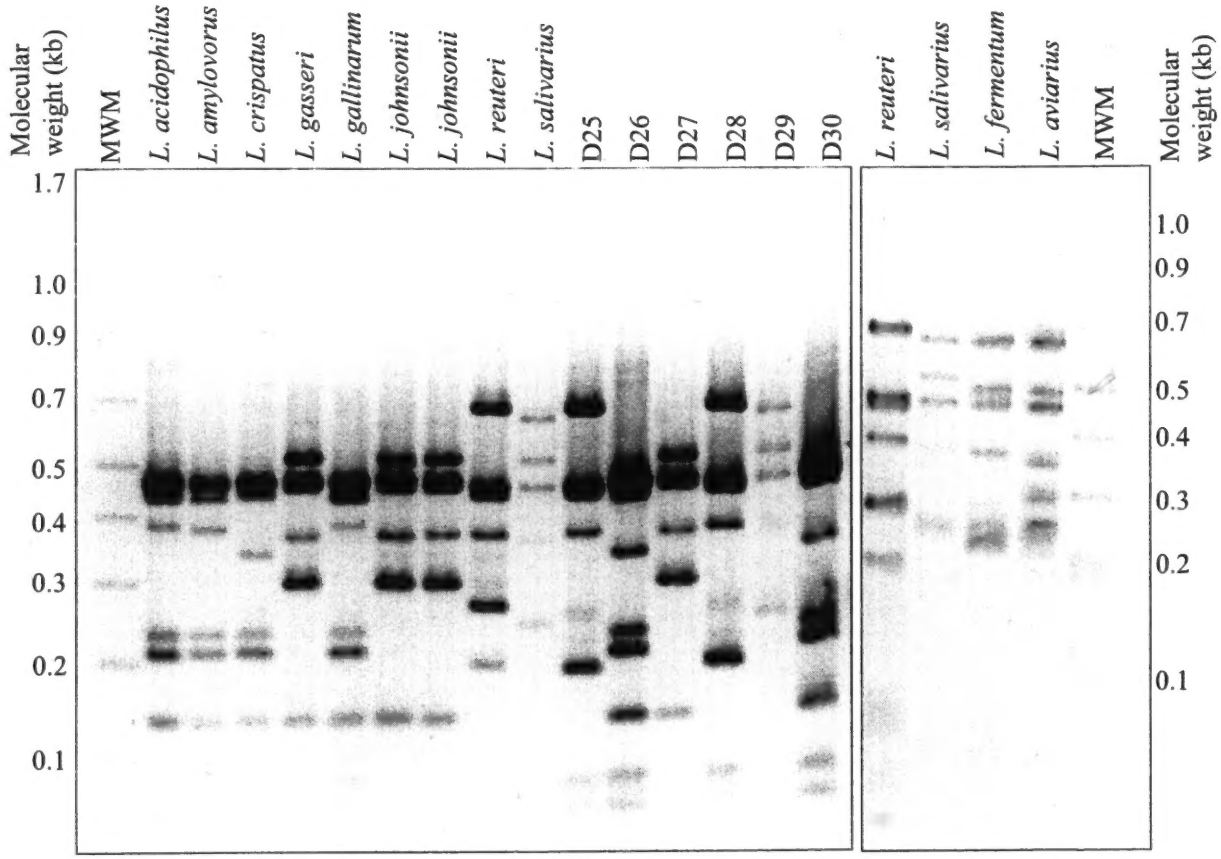

Fig. 2.7. *Hae*III-ARDRA profiles of 16S-23S rDNA from type and reference strains as well as crop isolates. MWM - molecular weight marker.

16-23S rDNA was used to make an initial identification of the crop isolates (Fig. 2.7 lanes D25-D30). Slight variations to the *Hae*III-ARDRA patterns were noted for *L. reuteri* (Fig. 2.7, compare banding pattern of the type strain with those of D25 and D28) and *L. crispatus* (data not shown) and were characterized by absence and/or differing intensity of bands between 250 bp to 300 bp for *L. reuteri* and between 300 bp to 400 bp for *L. crispatus*. The identity of the crop isolates with these "-like" patterns was confirmed by sequencing (refer to discussion, below and Table 2.4, above). The variability observed with these patterns is likely due to strain specific differences in the 16S-23S spacer regions [13,26].

In silico restriction mapping analysis using Lasergene software package by Leluo Guan indicated that *Mse*I could potentially generate species-specific patterns for the *L. acidophilus* group. Digestion of the 16-23S rDNA PCR products from the reference strains of the *L. acidophilus* group produced unique restriction patterns, with considerable variation between 200-850 bp (Fig. 2.8). All members of the *L. acidophilus* group gave unique patterns, which were used for the identification of isolates. The *Mse*I-ARDRA was therefore used to identify the crop isolates that had been previously placed in the *L. johnsonii/L. gasseri* and *L. amylovorus/L. gallinarum/L. acidophilus Hae*III-ARDRA groups. All the crop isolates from the *L. acidophilus* group were identified as *L. johnsonii*, *L. crispatus* or *L. gallinarum* (Table 2.8). As shown in Fig. 2.8, *Mse*I also generated unique patterns for *L. reuteri* ATCC 43272, *L. salivarius* subsp. *salivarius* ATCC 11741, *L. fermentum* ATCC14931 and *L. aviarius* ATCC 43234. Although *Mse*I could be used to identify these species among these isolates, we chose to use *Hae*III for initial screening because the banding pattern was simpler

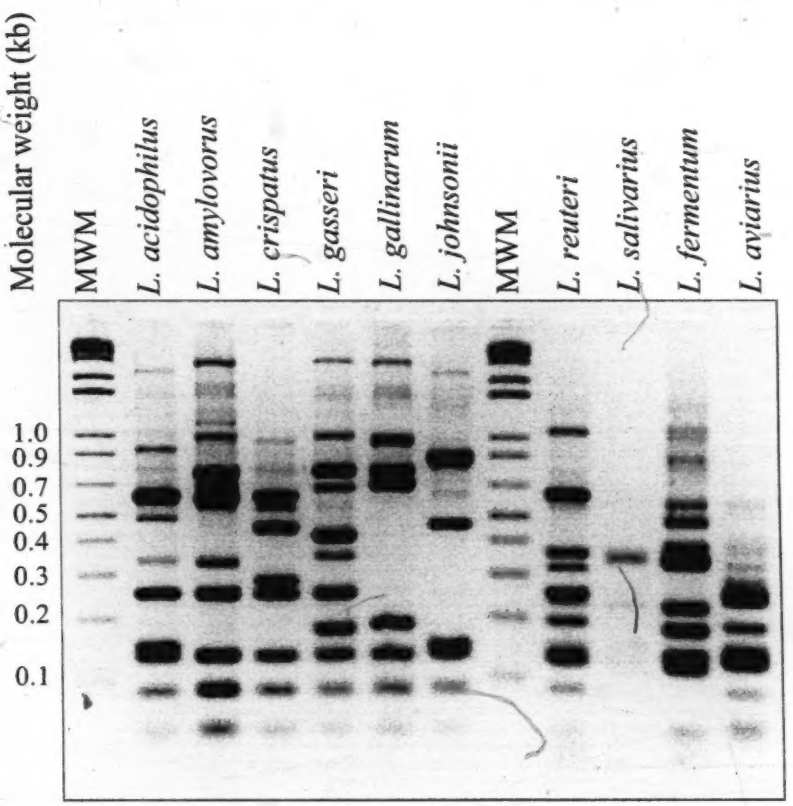

Fig. 2.8. *Mse*I-ARDRA profiles of 16-23S rRNA gene amplified from type strains of the *L. acidophilus* group as indicated (Table 2.2). MWM - Molecular weight marker.

Table 2.8. Identification of chicken crop isolates by *Hae*III- and *Mse*I-ARDRA and 16S rRNA gene sequence analysis.

No. of isolates	Characterization		Sequence analysis of the V2-V3 region of 16S rDNA in representative strain			
	*Hae*III-ARDRA	*Mse*I-ARDRA[1]	Strain no. (bp sequence obtained)	Species	Identity	GenBank Accession no.
73	*L. amylovorus* *L. gallinarum*	*L. gallinarum*	D64 (573)	*L. gallinarum*	99.5%	AJ417737
72	*L. crispatus*	ND	D139 (573)	*L. crispatus* ATCC33820	99.5%	AF257097
7	*L. crispatus-like*	ND	D68 (294)	*L. crispatus* ATCC 33820	98.9%	AF257097
35	*L. johnsonii*	*L. johnsonii*	D33 (581)	*L. johnsonii*	99.8%	M99704
	L. gasseri		14-1 (388)	*L. johnsonii*	99.3%	AJ002515
52	*L. reuteri*	ND	D15 (486)	*L. reuteri* DSM 20016 [T]	99.1%	X76328
				Lactobacillus spp.	98.6	AY005048
24	*L. reuteri-like*	ND	D3 (464)	*Lactobacillus* spp.	99.3	AY005048
				L. reuteri DSM 20016 [T]	98.6%	X76328
16	*L. salivarius*	ND	D29 (611)	*L. salivarius* subsp. *salivarius*	99.2%	AF335475

[1] ND - not tested
[T] Type strain

Two hundred and seventy-nine *Lactobacillus* crop isolates were identified to date using the *Hae*III- and *Mse*I-ARDRA methods, and the results are summarized in Table 2.8. The 16S rDNA sequences that were obtained from these isolates confirmed the species designation obtained by ARDRA (Table 2.8). Of the 279 *Lactobacillus* isolates, 78 (28%) were *L. crispatus,* 76 (27%) were *L. reuteri*, 35 (13%) were *L. johnsonii,* 73 (26%) were *L. gallinarum*, and 16 (6%) were *L. salivarius.* Twelve isolates generated *Hae*III-ARDRA patterns different from the reference lactobacilli and have not yet been identified.

2.4. Discussion

Earlier studies found that *L. salivarius*, *L. reuteri*, and *L. acidophilus* (old classification) inhabited the crop and that these species were present throughout the chicken digestive tract [21,32]. To our knowledge, ours is the first study utilizing nucleic acid-based techniques to investigate the composition of the *Lactobacillus* population in the crop throughout the development of broilers raised under commercial production conditions. The results showed that the crop microflora varied in composition during the life of the bird with some species, such as *L. acidophilus* and *L. salivarius,* appearing in a developmental succession while other species (i.e. *L. reuteri, L. johnsonii* and *L. crispatus/L. gallinarum/L. amylovorus*) were consistently detected. Sequence analysis of DGGE fragments demonstrated, for the first time, the presence of *L. johnsonii* and *L. crispatus/L. gallinarum* in the crop of broilers. With respect to the *L. acidophilus* group, analysis of individual Lac PCR-DGGE profiles from day 3 onwards indicated that *L. johnsonii* and *L. crispatus/L gallinarum/L. amylovorus* were present in over three quarters of the birds. The majority of isolates (Table 2.8) identified using ARDRA were *L. crispatus* and *L. gallinarum*, supplementing the PCR-DGGE data. In addition, no *L. acidophilus*, *L. amylovorus* or *L. fermentum* isolates were cultivated, suggesting that these species were absent or present in low numbers. Although the Lac PCR-DGGE could not distinguish between *L. fermentum, L. crispatus, L. gallinarum* and *L. amylovorus*, HDA PCR-DGGE showed that the PCR product amplified from *L. fermentum* had a unique migration distance (Fig. 2.4A) and a corresponding fragment was not observed in the pooled HDA PCR-DGGE profiles (Fig. 2.3). Individual profiles showed that only 4 out of 70 crops contained fragments that co-migrated with the

fragment from the *L. fermentum* reference strain. Thus, *L. fermentum* may be a member of the crop microflora only occasionally. Similarly, although Lac PCR-DGGE can distinguish *L. gasseri* from *L. johnsonii* (Fig. 2.4B), *L. gasseri* was not detected (Figs. 2.5 & 2.6 and data not shown), nor have any isolates been identified.

A rapid and simple ARDRA method was developed to distinguish between the members of the *Lactobacillus acidophilus* group. *Mse*I-ARDRA generated unique patterns for all species of the *L. acidophilus* group, identifying *L. crispatus*, *L. johnsonii*, and *L. gallinarum* among crop isolates (Table 2.8, Fig. 2.8). Ventura et al. [38] also described an ARDRA technique based on the amplification of the 16S rDNA only, followed by digestion with three enzymes, *Sau*3AI, *Hinf*I, and *Dra*I. *Sau*3AI digestion yielded group A- and group B-specific patterns, as well as differentiating among less related species of lactobacilli including *L. paracasei, L. salivarius, L. reuteri*, and *L. fermentum*. Following group designations provided by *Sau*3AI digestion, *Hinf*I produced a unique banding pattern for *L. acidophilus* and *L. gallinarum*, and *Dra*I could distinguish between *L. crispatus* and *L. amylovorus* as well as *L. johnsonii* and *L. gallinarum*. All three enzymes, however, were used to distinguish between the group A species; and two enzymes were required to identify the group B species. Roy et al. [29] proposed a combined group-/species-specific PCR followed by ARDRA. Group specific PCR was used to differentiate group A from group B *L. acidophilus*. A second PCR reaction was used to amplify the 16S rDNA and *L. acidophilus*/*L. amylovorus* were identified using *Hinf*I. Using an isoschizmer of *Mse*I, *Tru*9I, they distinguished between *L. crispatus*/*L. gallinarum* and *L. gasseri*/*L. johnsonii*. Thus, at least two PCR reactions and two restriction digests were required to identify the *L. acidophilus* group species. The

ARDRA used in our study simplified identification in that a single *Mse*I restriction digestion of one PCR product easily distinguished both closely related and less related lactobacilli. *Hae*III-ARDRA distinguished group A *L. acidophilus* from group B, differentiated *L. crispatus* from other group A *L. acidophilus*.

Based on PCR-DGGE and identification of the isolates, *L. crispatus* and *L. gallinarum* were consistently isolated from the chicken crop from day 0 (Fig 2.6). While *L. johnsonii* has been very well analysed and certain aspects of *L. crispatus* have been well studied [2,3,12,15,20,34,37], little information is available regarding *L. gallinarum*. The type strain of *L. gallinarum* was isolated from chicken crop [7], yet there have been no other studies of *L. gallinarum* and its interaction with its avian host to date. Very little is known about the characteristics that would promote its persistence in the gastrointestinal environment, although it has been proposed that S-layers of other group A *acidophilus* (GAA) may be involved in mediating adherence [2,20,34,37] (Chapter 1). Therefore, the focus of Chapter 3 is on identifying and characterizing the chicken crop *L. gallinarum* isolates and analysing their S-proteins.

Our study has provided detailed knowledge of the acquisition of the *Lactobacillus* microflora in the broiler crop. Of particular importance was the observation of the dynamics of the crop microflora during the life of the birds, demonstrating both rapid changes during days 1 to 7 and the establishment of a stable microflora after day 14. Further, since it has been proposed that the crop microflora acts as a bacterial inoculum for the remainder of the gut [9], knowledge of the composition of this bacterial collection is critical in understanding the contribution of the microflora members to the well-being of the avian host and for selection of species for probiotics. Given the crop

microflora dynamics observed in our study, it is doubtful that efficacious and scientifically valid probiotics can be derived without the use of this information because it impinges on the types of bacteria that will inoculate the digesta in the crop, suppress the multiplication of contaminating bacteria, and influence the biochemistry of the broiler gut [6,9]. The base-line information generated by this study will be essential in planning husbandry methods that utilize feed supplements other than antimicrobial drugs for the efficient production of broilers.

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Chapter 3: Molecular characterization of *L. gallinarum* crop isolates and analysis of their S-proteins

3.1. Introduction

The chicken crop has been shown to harbour a microflora dominated by *Lactobacillus* spp. [19], and lactobacilli have been shown to adhere to crop tissue [8,16-18]. Our culture-dependent and culture-independent investigation of the broiler chicken crop microflora (Chapter 2) revealed that *Lactobacillus gallinarum* is present in the crop in high numbers, as shown by both denaturing gradient gel electrophoresis (DGGE) data (Chapter 2 Figs. 2.5 & 2.6) and the ability to cultivate *L. gallinarum* isolates from crop homogenates at high dilutions (Chapter 2 Table 2.8). *L. gallinarum* is a member of the *Lactobacillus acidophilus* group, which is described in detail in Chapter 1 section 1.1.2. *L. gallinarum* and *Lactobacillus amylovorus* appear to be the least studied members of this group based on the paucity of published literature regarding these organisms. While the members of the *L. acidophilus* group are genetically and biochemically similar, each species may have a unique ecological niche. For example, *L. amylovorus* and *Lactobacillus gasseri* have not been isolated from the chicken GIT, while *Lactobacillus crispatus*, *Lactobacillus johnsonii* and *L. gallinarum* have [28] (Chapter 2, Table 2.8). The type strain of *L. gallinarum* was isolated from the chicken crop [15], however, little information regarding unique genetic and biochemical characteristics of *L. gallinarum*, and no information regarding its ecological niche in the chicken gastrointestinal tract (GIT), has yet been published.

The group A *acidophilus* (GAA) species are known to carry two genes for S-proteins, and DNA recombination resulting in differential S-protein expression in a subpopulation of *in vitro* grown *L. acidophilus* ATCC 4356 has been demonstrated [6] (Chapter 1 section 1.3.2.2). Therefore, it seems possible that phase variation of S-proteins could occur when GAA species experience are grown under different conditions (for examples see [12,13,21]), but differential expression of *Lactobacillus* S-proteins in the GIT has not been analysed to date. S-layers are thought to be important for adherence to surfaces, protecting the cell and allowing selective nutrient transport [38]. The N-terminus of mature GAA S-proteins ("variable regions") differ considerably and are predicted to contain domains for tissue binding and S-protein folding/S-layer formation [2,37,39]. Indeed, CbsA of *L. crispatus* JCM 5810 (a chicken isolate) was found to mediate collagen and laminin adherence [37] and has been demonstrated to adhere to *ex vivo* chicken colon tissue via unique sequences in the N-terminus [2,26,37]. The tissue structure and components in the crop and colon are different; the colon resembles intestinal epithelia, is collagen rich and coated with mucous while the crop epithelium is comprised of stratified squamous epithelia and is non-secretory [11]. Thus although collagen binding ability of *L. crispatus* JCM 5810 appears to be important for intestinal adherence, the adherence of crop isolates may be different. Therefore, the presence and characteristics of S-proteins from our crop *L. gallinarum* isolates could reveal information on the persistence of these strains in the chicken crop. Further, given that the S-proteins will be the outermost layer of the bacterial cell wall in the absence of bacterial capsules [38], it is possible that the S-layer may directly mediate adherence to the crop epithelium.

We analysed crop *L. gallinarum* isolates by determining their relatedness by genetic fingerprinting and then analysed the S-proteins expressed *in vitro* by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). We obtained 4 complete and 4 partial unique sequences for the variable regions of crop *L. gallinarum* S-protein genes, and used these sequences to screen the remaining *L. gallinarum* isolates for the presence of the gene types.

3.2. Materials and methods

3.2.1. Bacterial strains and growth conditions

3.2.1.1. *Lactobacillus gallinarum* crop isolates and *L. gallinarum* type strain

L. gallinarum cultures were routinely propagated on MRS agar (deMann, Rogosa, and Sharpe (Difco), Becton Dickinson, Sparks, MD; Cat. no. 0881-08) by streaking a loopful of liquid from the -80°C glycerol stock culture (as prepared in Chapter 2 section 2.2.2) and streaking to dilution. Where necessary, cultures were purified by restreaking to dilution the different colony types until only one colony morphology was observed on MRS agar. The cultures were then re-stocked into the glycerol culture collection and designated as *original culture name* #1, #2, etc. Standard growth conditions for *L. gallinarum* cultures were as follows: 37°C in an anaerobic environment (Thermo Forma anaerobic system with 5% CO_2, 10% H_2, balance N_2) for 48 hrs for plates and 24 hrs for broth unless otherwise stated. To propagate *L. gallinarum* isolates in liquid culture, a 5 to 10 mL aliquot of sterile MRS broth in a test tube was inoculated with from an MRS plate culture.

3.2.1.2: *Escherichia coli* transformants

E. coli JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_k-,m_k+), *rel*A1, *sup*E44, Δ(lac-proAB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15], [27]) were grown as follows: aerobically 37°C overnight with shaking (~150 revolutions per minute [rpm]) for broth (2YT, Luria Bertani [LB] or SOC) or in a 37°C incubator for plates (LB or MacConkey) unless otherwise stated. 2YT broth contained 1.6% Becton Dickinson Tryptone [w/v] [Franklin Lakes, NJ; Cat. no. 211921], 0.8% Becton Dickinson Yeast Extract [w/v] [Franklin Lakes, NJ; Cat. no. 211929] and 85.6 mM NaCl [33]. SOC broth contained 2%

Becton Dickinson Tryptone [w/v]; 0.5% Becton Dickinson Yeast Extract [w/v]; 8.56 mM NaCl and 20 mM Glucose [33]. LB broth contained 1% Becton Dickinson Tryptone [w/v], 0.5% Becton Dickinson Yeast Extract [w/v], 171mM NaCl [33]. LB agar contained 1% Becton Dickinson Tryptone [w/v], 0.5% Becton Dickinson Yeast Extract [w/v], 171mM NaCl and 1.5% agar [33]. MacConkey agar was purchased from Becton Dickinson (Difco, Cat. no. 212123).

3.2.2. Standard molecular biology techniques

Polymerase Chain Reaction (PCR) was always performed in a laminar flow cabinet. All PCR reagents, tips, and tubes were sterilized prior to use by autoclaving for 15 minutes at 121°C at 24 psi. With each PCR reaction, a negative control containing 1 µL of sterile MilliQ H_2O was used instead of the template DNA. PCR reagents and template DNA were stored at -20°C. PCR deoxynucleotide triphosphate (dNTP) mix was prepared from an Invitrogen (Burlington, ON) dNTP set (Cat. no. 10297-018), oligonucleotides were synthesized by Invitrogen and Recombinant Taq (*Thermus aquaticus*) Polymerase (Invitrogen Cat. No 10342-020) was used. PCR reactions were set up on ice, vortexed gently to mix, centrifuged briefly and placed into a pre-warmed GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA; Cat. no. 4134879).

For plasmid DNA extraction, genomic DNA extraction, Southern hybridization, FIGE (field inversion gel electrophoresis) and PFGE (pulse field gel electrophoresis), filter sterilized or autoclaved reagents were used where possible (sodium dodecyl sulphate [SDS] and NaOH solutions were not sterilized). Unless otherwise stated, solutions were sterilized by autoclaving. Sterile plastic tubes and baked glassware were used when needed. Agarose used for DNA analysis was purchased from Invitrogen (Cat.

no. 15510-027). Agarose gels were stained with 1 μg/mL ethidium bromide (EtBr, Bio-Rad, Hercules, CA; Cat. no. 161-0433). Agarose for DNA analysis was supplied by Invitrogen (Burlington, ON; Cat. no. 15510-027). Acrylamide for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was purchased from Bio-Rad (Cat. no. 161-0148). Images were captured with an Alphadigidoc system (AlphaInnotech, San Leandro, CA; Cat. no. AD-1201-1) using a UV-transilluminator for agarose gels and a white light pad for SDS-PAGE gels.

Alignments for S-protein/S-protein gene sequences were performed with ClustalW using either the MegAlign module of the Lasergene software package (DNAStar Inc, Madison, WI) or the ClustalW online alignment tool (http://www.ebi.ac.uk/clustalw/) with the default settings. When appropriate, ClustalV was used in MegAlign.

3.2.3. Species identification of *L. gallinarum* isolates

L. gallinarum isolates were identified using ARDRA (amplified ribosomal DNA restriction analysis) as outlined in Chapter 2 section 2.2.7.

Partial 16S rDNA sequencing was conducted on two selected isolates (D109 and D195#2) by Leluo Guan to confirm the species designation using primers Lb16a and Lac2. PCR reactions were performed using the following program: 94°C for 5 min; 25 cycles of 94°C for 45 sec, 53°C for 30 sec, and 72°C for 1 min 30 sec; and concluded with a final extension at 72°C for 7 min. Each PCR reaction contained 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 1 pmol/μL each oligonucleotide (Lb16a and Lac-2, Chapter 2 section 2.2.7) and 0.025 U/μL Taq Polymerase and 1 μL dilute (1/20) template DNA. Aliquots (10 μL) of the PCR products and the appropriate oligonucleotide (diluted to 5

pmol/μL) were stored at -20°C until given to the Agricultural, Food and Nutritional Science Biotech Core, University of Alberta, for sequencing.

3.2.4. Strain identification and groupings

3.2.4.1. Genetic fingerprinting of *L. gallinarum* isolates using Pulsed Field Gel Electrophoresis

In order to genetically fingerprint *L. gallinarum* isolates and identify strains present, PFGE plugs were prepared using the protocol of Tanskanen et al. [40] with the modifications outlined below. *L. gallinarum* cultures were streaked on MRS plates (in some instances, time did not permit for full 48 hours incubation). One to four colonies were used to inoculate 9-10 mL sterile MRS broth, and grown overnight. The following morning, 1% of the overnight culture volume was transferred into 9-10 mL sterile MRS broth. When the culture had reached an optical density at 600 nm (OD_{600}) between 0.3 and of 0.6, chloramphenicol (10 mg/mL made in 95% ethanol and stored at -20°C) was added to give a final concentration of 100 μg/mL and the culture was incubated for 1 hr. The OD_{600} at which the chloramphenicol was added determined the volume of cell suspension that was used for plug preparation as follows: at an OD_{600} of 0.6, 1.5 mL was used; and at an OD_{600} of 0.3, 8-9 mL were used. The cells were centrifuged at 3900xg in a 15 mL BlueMax Jr Falcon tube (BD Cat. no. 352097) and washed in 1.5-3 mL of cell wash buffer (CWB, 1M NaCl; 10 mM Tris-HCl), then resuspended in 200-300 μL of CWB. The cell suspension was then transferred to sterile 1.7 mL microfuge tubes and an equal volume of 2% low melting point agarose (Invitrogen Cat. no. 155 17014, made in CWB and equilibrated to 50°C) was added to the tube and was mixed by pipetting, transferred into 3 to 5 plug moulds (Bio-Rad Cat. no. 170-3622) and placed at -20°C for at least 5 min. The plugs were carefully pushed out of the plug moulds into 2 mL of

proteinase K buffer solution (0.25M EDTA [ethylene diamine tetra acetic acid, pH 8.0]; with 1% N-lauryl sarcosine [w/v] and 100 μg/mL proteinase K [Invitrogen Cat. no. 25530-031]) per plug and incubated at 50°C overnight. Plugs were washed at least 4 times for 1 hr in 10-15 mL sterile 1X TE (Tris EDTA, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) the following day and then placed in 2 mL sterile lysis buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA [pH 8.0]) per plug containing 1 mg/mL lysozyme and 40 activity units/mL of mutanolysin (Sigma-Aldrich, Oakville ON; Cat. no. M9901). The plugs were incubated at 37°C overnight. The following day, the plugs were treated again with proteinase K using the same conditions described above. Following the final proteinase treatment, the plugs were washed in 1X TE as outlined above and placed in storage buffer (0.5 M EDTA and 1% N-lauryl sarcosine [w/v]) until use.

When plugs were prepared for electrophoresis, they were washed as above in 1X TE and sliced into 1 mm thick slices using two flat-edged razor blades held together with a paperclip. One to three slices were placed in a sterile 1.7 mL microfuge tube containing 100 μL restriction enzyme digest mixture (60U *Sma*I [New England Biolabs, Pickering ON Cat. no. R0141L] in 1X NEBuffer 4 [50 mM KOAc, 20 mM TrisOAc, 10 mM $Mg(OAc)_2$, 1mM dithiothreitol {DTT}, pH 7.9] or 30 U *Apa*I [New England Biolabs, Cat. no. R0114L] with 0.1 μg/mL bovine serum albumin [BSA] in 1X NEBuffer 4). Digests were incubated at room temperature overnight. The following day, the 15 cm wide agarose gel mould was assembled and levelled on a flat surface. The plug slices were carefully placed on the front of the 15 well comb with the cut side facing "up" (i.e. the cut side was placed perpendicular to the direction of the current). Markers (Lambda Ladder PFGE Marker [New England Biolabs Cat. no. N0340S] and Low Range PFGE

Marker [New England Biolabs Cat. no. N0350S]) were also sliced and placed on the comb. Excess buffer was removed from around the plug slices with a pipette, the comb was slotted vertically into the mould, and 100 mL of 1% agarose (Invitrogen Cat. no. 15510-027) in 0.5X TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA, equilibrated to 50°C) was quickly and steadily poured. While the agarose solidified, the running buffer (2.2 L of 0.5 X TBE) was prepared and chilled to 14°C in the electrophoresis chamber by circulating through the Bio-Rad CHEF Mapper XA chiller. After the agarose solidified and the running buffer cooled, the gel was placed in the electrophoresis unit. A Bio-Rad CHEF Mapper XA Pulsed Field Electrophoresis System (Cat. no. 170-3670) was connected to the electrophoresis unit and the "Auto Algorithm" program, set to separate 20-200 kb, was selected (6.0 V/cm, initial switch time of 2.98 sec, final switch time of 17.33 sec, linear ramping factor, 120° angle). Upon completion of the run (26 hrs and 56 min), the gel was removed from the electrophoresis unit and stained in EtBr in 1X TBE for 15-30 minutes and destained 10-30 minutes in 0.5 XTBE. Agarose gels were analysed with Bionumerics (Applied Maths, Austin, TX) using the Dice's similarity coefficient (D_{sc}) with Ward dendrogram using fuzzy logic and 6% position tolerance.

3.2.4.2. Grouping of *L. gallinarum* isolates using Randomly Amplified Polymorphic DNA (RAPD)

Oligonucleotides OPA-02 and OPA-18 [32] were used separately in RAPD-PCR reactions. PCR was performed with the following cycling conditions: 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 32°C, 2 min at 72°C; and a final 7 min hold at 72°C. Each reaction contained 0.25 mM dNTP mix, 2.5 mM $MgCl_2$, 0.25 pmol/μL each oligonucleotide, 0.125 U/μL Taq Polymerase and 2% dilute (1/20) template DNA [v/v]

(Chapter 2 section 2.2.4) in a 20 μL or 50 μL PCR reaction as needed. Fifteen microlitres of the RAPD-PCR reaction was loaded on a 1.5-2% agarose gel. Agarose gels were analysed with Bionumerics using the Dice's similarity coefficient (D_{sc}) with Ward dendrogram using fuzzy logic and 5% position tolerance. Isolates producing the same or similar banding patterns were grouped into the same RAPD group.

3.2.4.3. Comparison of the plasmid profiles of *L. gallinarum* isolates

Plasmid DNA was isolated from *L. gallinarum* crop isolates using the protocol of Walker et al. [44] with the modifications outlined. *Lactobacillus* cultures streaked on MRS agar plates were inoculated into 9-10 mL sterile MRS broth and grown overnight anaerobically at 37°C. Two millilitres of overnight culture was used to inoculate 9-10 mL of sterile MRS broth and the culture was grown under the same conditions for approximately 2 hrs. The entire culture was pelleted in a 15 mL BlueMax Jr Falcon tube by centrifugation at 3900xg for 5 minutes, the pellet was washed with 5 mL cold sterile MilliQ H_2O, and centrifuged again as above. The cell pellet was resuspended in 1 mL sterile cell suspension buffer (50 mM Tris-HCl, 1 mM EDTA and 8% sucrose [w/v] [filter sterilized]; 10 mg/mL lysozyme was added just before use) and the cells were incubated on ice for 1 hr. The cells were transferred to a microfuge tube and centrifuged for 1 min at 3900xg. The supernatant was removed, and the cell pellet was resuspended in 500 μL lysis solution (50 mM Tris-HCl, 5 mM EDTA, 3% SDS [w/v] [filter sterilized]), which was pH adjusted with 35 μL/mL of freshly prepared 3 M NaOH just prior to use. The pellet was disrupted by the use of a pipette and the cells were heated to 65°C for 30 min and then cooled to room temperature. Two hundred and fifty microlitres of high salt solution (3 M KOAc with 1.8% glacial acetic acid [v/v] [filter sterilized]) was

added to each tube and mixed by vortexing or inverting. Four hundred microlitres of TE-saturated phenol (Invitrogen Cat. no. 15513-047) and 400 μL of chloroform were added to each tube. The tube was vortexed gently and centrifuged at 14800xg for 5 min. The aqueous (upper) layer was transferred into a new sterile microfuge tube, 750 μL of chloroform:isoamyl alcohol (24:1) was added, and the tube was mixed. The tubes were centrifuged as above and the aqueous (upper) layer was transferred into a sterile microfuge tube to which 750 μL of cold isopropyl alcohol was added and then mixed. The tubes were placed at -20°C for a minimum of 30 min and then centrifuged at 14600xg for 15-20 min. The supernatant was discarded and the DNA pellet was washed with 70% DNA grade ethanol, dried, and resuspended in 20 μL of sterile MilliQ H_2O containing 20 μg/mL of RNase A. Ten microlitres of the plasmid preparation was loaded on an 0.8% agarose gel, run for 16-20 hrs at 20 V, and then visualized by staining with EtBr. Agarose gels were analysed with Bionumerics using the Dice's similarity coefficient (D_{sc}) with Ward dendrogram using fuzzy logic and 5% position tolerance.

3.2.5. Detection and Characterization of S-proteins and corresponding genes

3.2.5.1. S-protein extraction and characterization

S-proteins were extracted using a protocol modified from Chagnaud et al. [10]. MRS broth (5 mL) was inoculated with a plate culture (in some instances, time did not permit for full 48 hours incubation) and incubated overnight (approximately 16-20 hours). The test tube was vortexed and 1 mL of the culture was aliquoted into 4 x 1.7 mL microfuge tubes. The tubes were centrifuged at 4500xg for 5 min, and the cell pellets were washed twice with sterile 0.85% NaCl [w/v] following the same centrifugation settings as outlined above. The pellets were resuspended in 100 μL of 0.01 M Tris-HCl

(pH 8.8) to which 100 µL of extraction buffer (0.08 M Tris-HCl [pH 6.8] with 1% β-mercaptoethanol [v/v] and 2% SDS [w/v]) was added and mixed by vortexing. The tubes were incubated at 70°C in a drybath for 10 min, cooled to room temperature and centrifuged at 16000xg for 5 min. The supernatants were collected and pooled for each isolate, and the pellets and supernatants were stored at -20°C until further use.

To detect the presence of presumptive S-proteins from *Lactobacillus* isolates, the supernatant was analysed using SDS-PAGE. The supernatant was thawed and vortexed, and 5 volumes of supernatant sample was added to 1 volume of 6X SDS-PAGE loading dye (0.00625 M Tris-HCl pH 6.8, 60% glycerol [v/v], 2% SDS [w/v] and 0.5% β-mercaptoethanol [v/v]). Five to thirty microlitres of each S-protein extract was used, as needed. Ten microlitres of prestained SDS-PAGE molecular weight standards (Bio-Rad Prestained Broad Range SDS-PAGE standards Cat. no. 161-0318 or Sigma Wide Range Colour Markers Cat. no. C 3437) were aliquoted into 1.7 mL microfuge tubes. The standards and samples were boiled or heated to 95°C in a drybath for 4 min and kept at room temperature until loading. A 1 mm thick 15% resolving SDS-PAGE (15% acrylamide [37.5 acrylamide:1 bis-acrylamide] [v/v], 0.1% SDS [w/v], 0.375 M Tris-HCl pH 8.8, with 50 µL 10% APS [w/v] and 5 µL TEMED [N,N,N',N'-Tetramethylethylenediamine] added per 10 mL to catalyze polymerization) was prepared in a Bio-Rad Mini-PROTEAN 3 system (Cat. no. 1653301) and topped with a 4% stacking gel (4% acrylamide [37.5:1] [v/v], acrylamide, 0.1% SDS [w/v], 0.125 M Tris-HCl pH 6.8, with 50 µL 10% APS [w/v] and 10 µL TEMED added per 10 mL to catalyze polymerization). The sample was loaded into the wells and run in 1X SDS-PAGE running buffer [pH 8.3] (0.025M Tris-HCl, 0.192M glycine, 0.1% SDS [w/v]) at 100-150

V until the dye front was near the bottom of the gel. The gel was washed three times with MilliQ H_2O for 5 min, stained with Sigma EZBlue staining reagent (Sigma Cat. no. G 1041) for 30 min, then washed several times in MilliQ H_2O to remove background and destained for at least 40 min prior to image capture and analysis. Bionumerics was used to estimate S-protein molecular weight within and between gels. The Dice's similarity coefficient (D_{sc}) with Ward dendrogram and 2% position tolerance was used when comparing data from gel to gel.

3.2.5.2. Detection and Amplification of S-protein genes from *L. gallinarum* using PCR

Primers were designed using S-protein gene and protein sequence alignments analysed with the Lasergene software package. Alignments for S-protein genes from *L. acidophilus* and *L. crispatus* (GenBank accession numbers X89375, X89376, AF001313 AF079365, AJ007839, AF253043 and AF253044, Chapter 1 Table 1.1) were used to determine conserved regions where degenerate primers could be designed (Fig. 3.1). A degenerate primer pair, Usl-1 and Usl-2 were designed to amplify both S-protein genes (Table 3.1). A single 20 µL PCR reaction contained 0.2 mM dNTP mix, 1 mM $MgCl_2$, 1 pmol/µL each oligonucleotide and 0.05 U/µL Taq Polymerase. One microlitre of a 1/20 [v/v] dilution of total DNA extracted from *Lactobacillus* isolates was used as template in each reaction. The samples were exposed to a touchdown PCR program consisting of the following: 5 min at 94°C; 2 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 1 min; 2 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 1 min; 2 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 1 min; 30 cycles of 94°C for 30 sec, 60°C for 30

A.

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Usl-1                                        GAATYGTKAGCGCTSCTGCTGC              22
L. acidophilus ATCC 4356 slpA  ATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGCTGCTTTACTTGC 50
L. acidophilus ATCC 4356 slpB  ATGAAGAAAAATTTAAGAATCGTTAGCGCTGCTGCTGC---TTTACTTGC 47
L. crispatus JCM 5810 cbsA     ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
L. crispatus JCM 5810 cbsB     ----------------------GTGAGCGCTCCTGCTGCTGCTTTATTAGC 29
L. crispatus LMG 12003 slpnA   ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
L. crispatus LMG 12003 slpnB   ATGAAGAGAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
L. crispatus M247 s-layer      ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
L. crispatus MH315 lbsA        ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
L. crispatus MH315 lbsB        ATGAAGAAAAATTTAAGAATTGTTAGCGCTGCTGCTGCTGCTTTATTAGC 50
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B.

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L. acidophilus ATCC 4356 slpA  GTACTAAGCGTACTTTGAAGCACAACGCTTACGTTTACGCATCATCAAAG 1191
L. acidophilus ATCC 4356 slpB  GTACTAAGCGTACTTTGAAGCACAACGCTTACGTTTACGCATCATCAAAG 1227
L. crispatus JCM 5810 cbsA      GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACAAGTCTTCAAAG 1182
L. crispatus JCM 5810 cbsB      GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACAAGACTTCAAAG 1218
L. crispatus LMG 12003 slpnA    GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACAAGACTTCAAAG 1236
L. crispatus LMG 12003 slpnB    GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACAAGTCTTCAAAG 1179
L. crispatus M247 s-layer       GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACAAGACTTCAAAG 1215
L. crispatus MH315 lbsA         GTACTAAGCGTACTTTGAAGCACAACGCATACGTTTACGCAACTTCAAAG 1260
L. crispatus MH315 lbsB         GTACTAAGCGTACTTTGAAGCATAACGCATACGTTTACAAGTCTTCAAAG 1188
Usl-2 (RC)                                  GAAGCACAACGCWTACGTTTAC              22
                                            ****** ***** *********
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Fig. 3.1. Position of **A**. Usl-1 and **B.** reverse complement of Usl-2 oligonucleotide primers for amplification of S-protein genes in *L. gallinarum*. "*" indicates identical nucleotide in all sequences. The GenBank accession numbers are as follows: ATCC 4356 *slp*A, X89375; ATCC 4356 *slp*B, X89376; JCM 5810 *cbs*A, AF001313; JCM5810 *cbs*B, AF079365; LMG 12003 *slpn*A, AF253043; LMG 12003 *slpn*B, AF253044; M247 *s-layer*, LCR7839; MH315 *lbs*A, AB110090; MH315 *lbs*B, AB110091.

Table 3.1. Oligonucleotides used to amplify and/or sequence the S-protein genes of *L. gallinarum* isolates

Primer name[1]	Sequence (5' to 3')	Alignment	PCR partner	Tm (°C)
T7	AATACGACTCACTATAGG	Vector sequence	Sp6	45
Sp6	AATTAGGTGACACTATAG	Vector sequence	T7	
Usl-1	GAATYGTKAGCGCTSCTGCTGC	nt 17-38 of the reading frame of *cbs*A from *L. crispatus* JCM 5810 (AF001313)	Usl-2	
Usl-2	GTAAACGTAWGCGTTGTGCTTC	nt 1170-1149 of the reading frame of *cbs*A from *L. crispatus* JCM 5810 (AF001313)	Usl-1	Touchdown from 63 to 60
Usl-3	CATACTTCTACGACAAGGACGC	nt 965-986 of the reading frame of *cbs*A from *L. crispatus* JCM 5810 (AF001313)	Usl-2	
Fsl-1	CATCAAACAATACTGTTACAAAC	nt 417-429 of complete clone D109D sequence	Fsl-2	51
Rsl-1	CAAACCATTAGCATCAATATC	nt 760-740 of complete clone D109D sequence	Fsl-1	
D109D-1	GCAACTTGGTTCAAGGTTACAGTAC	nt 492-468 of complete clone D109D sequence	Usl-1	Touchdown from 63 to 60
D109D-2	CATACAGCAACAACATCAAAGAAGC	nt 696-720 of complete clone D109D sequence	Usl-2	
Fsl-2	GATAACGGATCACACTTTGG	nt 407-426 of complete clone D1952F sequence	Rsl-2	53
Rsl-2	AGGTTGTTAATGTTAGCTGTAG	nt 741-720 of complete clone D1952F sequence	Fsl-2	

[1]Grey fill – primers used for screening in section 3.2.5.4
[2]K represents T or G, W represents A or T, Y represents C or T

Table 3.1. Continued

Primer name[1]	Sequence (5' to 3')	Alignment	PCR partner	Tm (°C)
D1952F-1	GGCAGTTACAGGGAAGATTACGTATG	nt 517-492 of complete clone D1952F sequence	Usl-1	Touchdown from 63 to 60
D1952F-2	GTTATTTCAGCTGTTAGCCCATTAG	nt 638-662 of complete clone D1952F sequence	Usl-2	
Fsl-3	CAAAGGCTGAAATGACTACTAC	nt 504-525 of complete clone D1952C sequence	Rsl-3	57
Rsl-3	CTTAGCATTGTCAACATGTACG	nt 1102-1081 of complete clone D1952C sequence	Fsl-3	
D195 2C-1	CTAAGTCACCTGAAGTAGTACC	nt 770-749 complete clone D1952C sequence	Fsl-3	57
D195 2C-2	GCATACGGCAACAGTTATGAC	nt 863-883 complete clone D1952C sequence	Rsl-3	
D1952C-3	CAAAGTCACCGTTTGAAGCACG	nt 590-569 of complete clone D1952C sequence	Usl-1	Touchdown from 63 to 60
D1952C-4	ACGGTAAGGTTGCAGGTCATG	nt 960-979 of complete clone D1952C sequence	Usl-2	
Fsl-5	CAACTTGACAAGAATGGTACTG	nt 467-488 of complete clone D256A sequence	Rsl-5	57
Rsl-5	GTACTTGATGTCACTTGATTCC	nt 724-703 of complete clone D256A sequence	Fsl-5	
D256A-1	GGGTTAGCAAAGTCCTTAGCAG	nt 540-519 of complete clone D256A sequence	Usl-1	Touchdown from 63 to 60
D256A-2	CAAGTTATTGCAGCTGTTCGTG	nt 641-662 of complete clone D256A sequence	Usl-2	

[1]Grey fill – primers used for screening in section 3.2.5.4

[2] K represents T or G, W represents A or T, Y represents C or T

Table 3.1. Continued

Primer name[1]	Sequence (5' to 3')	Alignment	PCR partner	Tm (°C)
Fsl-4	GTAGGTTTGAACTTTGGTACTC	nt 386-407 of 5' end of partial D255B sequence	Rsl-4	55
Rsl-4	AACATCAATGTTCATAGCCTTC	nt 51-30 of 3' end of partial clone D255B sequence	Fsl-4	
Fsl-6	GTTACTATCACTGTTCCAGCAGG	nt 365-385 of 5' end of partial clone D44#2B sequence	Rsl-6	63
Rsl-6	CTACGTAGTTCTTATCAAATTCAG	nt 78-55 of 3' end of partial clone D44#2B sequence	Fsl-6	
Fsl-7	GGGCTGCACATAATGCGACTGA	nt 414-435 of 5' end of partial clone D42C sequence	Rsl-7	63
Rsl-7	GAAATAGCACTTGCATCTGAGCC	nt 185-163 of 3' end of partial clone D42C sequence	Fsl-7	
D109E-1	GTTAACGTCTTGCAACATACTTAG	nt 165-142 of 5' end of partial clone D109E sequence	Usl-1	Touchdown from 63 to 60
D109E-2	CATTAACTTGGGTGGTTCAGACG	nt 116-139 of 5' end of partial clone D109E sequence	D109E-3	63
D109E-3	TACCGCCTTGTACAGCAGTAACC	nt 364-343 of 5' end of partial clone D109E sequence	D109E-2	

[1] Grey fill – primers used for screening in section 3.2.5.4

[2] K represents T or G, W represents A or T, Y represents C or T

sec and 72°C for 1 min; and 72°C for 7 min. PCR products (1.1-1.2 Kb) were analysed on a 1% agarose gel.

3.2.5.3. Cloning and sequencing of S-protein gene

The *L. gallinarum* S-protein PCR products were cloned into *E. coli* JM109 using the pGEMT-Easy (Promega, Madison WI; Cat. No A1380) kit. Usl1/Usl-2 PCR products were purified using the Qiaex II kit (Qiagen, Mississauga, ON; Cat. no. 20021) following the manufacturer's protocol for desalting and concentrating DNA solutions. Purified PCR product (1.5 μL) was ligated to 25 ng of pGEMT-Easy vector in a 5 μL ligation reaction containing 1.5 U T4 DNA ligase in 1X ligase buffer (30 mM Tris-HCl [pH7.8], 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP [adenosine triphosphate]). The tubes were gently mixed by pipetting and centrifuged briefly. The ligations were incubated at 4°C overnight. $RbCl_2$-competent *E. coli* were prepared by Leluo Guan and Teresa Grayson using the procedure of Hanahan [20] with the following modifications by Robert Hallewell (Chiron Corporation): 100 mL prewarmed LB broth was inoculated with 1% [v/v] of an overnight culture and grown with vigorous aeration (200-250 rpm) until an optical density at 550 nm [OD_{550}] of 0.48 was reached. The cells were chilled and centrifuged for 5 min at 5000xg, then resuspended in 30 mL transformation buffer I (30 mM KOAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% (w/v) glycerol, pH 5.8, filter sterilized). The cells were incubated on ice for 2 hrs and then centrifuged at 5000xg and resuspended in 4 mL transformation buffer II (10 mM NaMOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol [w/v], filter sterilized, pH 7.0). The cells were aliquoted in 100 μL fractions and stored at -80°C until use. Two microlitres of the ligation reaction was transferred into a sterile 1.7 mL microfuge tube to which 40 μL of $RbCl_2$ competent *E.*

coli were added. The tube was mixed by flicking and placed on ice for 20 min. The cells were heat shocked at 42°C for 45-50 sec and placed on ice for 2 min. Transformed *E. coli* were grown in 950 µL of sterile SOC medium for 1.5-3.5 hrs. Aliquots of 10 and 100 µL from the culture were plated on to MacConkey plates containing 100 µg/mL ampicillin or LB plates containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and IPTG (isopropyl-beta-D-thiogalactopyranoside) at 100 µg/mL, 80 µg/mL and 0.5 mM, respectively. Pale pink colonies (MacConkey) or white colonies (LB+X-Gal+IPTG) were picked and restreaked onto the same agar media used to propagate the transformants. Colonies picked from the plates were inoculated into 2 mL of 2YT broth containing 100 µg/mL ampicillin and grown for 8 hrs or 24 hrs.

A maximum of eight *E. coli* clones were picked for each ligation. For each clone, 1.5 mL of cells was used for a plasmid extraction, and the remaining 500 µL was transferred into a screw capped tube with 1 mL LB (1% Bacto Tryptone [w/v]; 0.5% Bacto Yeast Extract [w/v] and 0.17 M NaCl) containing 50% glycerol [v/v], and frozen at -80°C. The alkaline plasmid extraction protocol [33] was applied to the 1.5 mL of culture. Briefly, cells were pelleted by centrifuging for 3 min at 12500xg and the supernatant was discarded. One hundred microlitres of sterile GTE (50 mM Glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) was used to resuspend the cell pellet, followed by the addition of 200 µL of lysis solution (0.2M NaOH 1% SDS [w/v]). The mixture was vortexed gently and incubated on ice for 10 min. One hundred and fifty microlitres of potassium acetate solution (3M KOAc, 11.5% glacial acetic acid [v/v]) was added to each tube and the tubes were mixed as before and incubated on ice for 10 min. The tubes were centrifuged for 5 min at 12500xg and the supernatant was transferred into

a new tube. The plasmid DNA was precipitated by adding 900 µL cold 95% DNA grade ethanol and left at -20°C overnight. The plasmid preparations were then centrifuged at 14600xg for 20 min at 4°C, The DNA pellets were dried and resuspended in 500 µL of sterile MilliQ H_2O. RNase A was added to a final concentration of 0.1 mg/mL and the DNA was incubated at 37°C for 2 hrs. The plasmid DNA was then phenol extracted by the addition of 500 µL TE saturated phenol, vortexed, and centrifuged at 14600xg for 3 min. The upper (aqueous) layer was removed and placed in a sterile 1.7 mL microfuge tube with 500 µL chloroform. The tube was vortexed and centrifuged as above. The DNA was precipitated by the addition of 50 µL NaOAc and 1 mL of cold 95% ethanol. The DNA was precipitated at -20°C overnight and centrifuged the following day at 4°C for 20 min at 14600xg. The pellet was dried and resuspended in 50 µL sterile MilliQ H_2O.

PCR with Usl-1/Usl-2 primers, as described in section 3.2.5.2, was used to confirm the presence of the PCR product in recombinant plasmids. Typically, 1 µl of a 1/10 dilution of plasmid DNA was used as template in the PCR reaction for clones grown 24 hrs. For clones grown for only 8 hrs, 1 µl of undiluted plasmid DNA was used as template. Only those plasmids producing a PCR product in the correct size range were characterized further by sequencing.

Sequencing was performed as follows. A 50 µL PCR reaction was set up containing 0.2 mM dNTP mix, 2 mM $MgCl_2$, 0.5 pmol/µL each oligonucleotide (T7 and Sp6, Table 3.1) and 0.05 U/µL Taq Polymerase. Each reaction contained 1 µL diluted or undiluted plasmid DNA (as described above). The PCR reactions were conducted as follows: heating to 94°C for 3 min followed by 25 cycles of 30 sec at 94°C, 45 sec at 45°C (Table 3.1) and 1 min of extension at 72°C, then a hold at 68°C for 7 min. Products

were aliquoted (10 μL) into sterile tubes. Products and the appropriate oligonucleotide (diluted to 5 pmol/μL) were stored at -20°C until given to the Agricultural, Food and Nutritional Science Biotech Core, University of Alberta, for sequencing.

Primer walking was performed to complete the sequence of the variable region of the S-protein gene of various isolates. Oligonucleotides were designed based on sequences obtained from clones and are listed in Table 3.1. The oligonucleotides were then used in a 25 μL PCR reaction (0.2 mM dNTP mix, 1 mM $MgCl_2$, 1 pmol/μL each oligonucleotide and 0.05 U/μL Taq Polymerase) with the following conditions: heating to 94°C for 5 min followed by 30 cycles of 30 sec at 94°C, 30 sec at the oligo-specific melting temperature (Tm, Table 3.1) and 1 min of extension at 72°C, then a hold at 72°C for 7 min. The PCR products (2.5 μL) were examined on a 1% agarose gel while the remainder was submitted for further sequencing as described above.

Sequences were assembled in the SeqmanII module of the Lasergene software package and the consensus was determined by comparing sequences from both DNA strands. In cases of disagreement, the chromatograms were examined and compared. The validity of the sequence was verified by examining the translation in the +2 frame for the absence of cysteine residues [38] and stop codons. S-protein genes were completely or nearly completely sequenced in both directions. In some cases, short areas of good quality single stranded sequence was used to complete the sequence.

3.2.5.4. Screening of *L. gallinarum* isolates using S-protein gene specific primers

L. gallinarum isolates were screened for the presence of unique S-protein gene types using PCR containing predicted S-protein specific oligonucleotide pairs highlighted in Table 3.1. PCR was performed as above for sequencing. One positive and two negative

controls were included each time a PCR set was done. The negative controls consisted of a) 1 µL sterile MilliQ H_2O and b) 1 µL dilute (1/20) *L. johnsonii* ATCC 33200 DNA, and the positive control consisted of 1 µL 1/20 dilute DNA of the isolate the gene type was cloned from (e.g. D109 for Fsl-1/Rsl-1, Table 3.1).

3.2.5.5. Southern hybridizations

Agarose plugs containing *Lactobacillus* DNA used for PFGE (section 3.2.4.1) were used in FIGE to separate small DNA fragments (1-10 kb). The agarose plugs were washed in 1X TE and sliced as described in section 3.2.4.1. One or two slices were placed in a sterile 1.7 mL microfuge tube containing 100 µL enzyme digest mixture (20U *Eco*RI in 1X REact 3 buffer [50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl]). Digests were incubated at 37°C overnight. The following day, the 20 cm wide agarose gel mould was assembled, with the 30 well comb loaded as described in section 3.2.4.1. The Low Range PFGE Marker was used. Two hundred and fifty millilitres of 1% agarose in 0.5X TBE (equilibrated to 50°C) was quickly and steadily poured. While the agarose solidified, the running buffer was prepared and chilled as above (section 3.2.4.1) The agarose gel was placed in the electrophoresis chamber and the FIGE program was used with an initial switch time of 0.06 sec, a final switch time of 0.08 sec, a forward voltage gradient of 9 V/cm, a reverse voltage gradient of 6 V/cm. Upon completion of the run (17 hrs and 29 min), the gel was removed from the electrophoresis unit and stained in EtBr in 1X TBE for 15-30 minutes and destained 10-30 min in 0.5 X TBE.

Probes for Southern hybridization (Table 3.2) were prepared by PCR of *L. gallinarum* template DNA with S-protein gene type specific oligonucleotides under the conditions listed in Table 3.2. For the universal probe, Usl-1/Usl-2 PCR using dilute

Table 3.2. Probes, hybridization and washing temperatures, and exposure times used for Southern hybridizations

Probe name	Target sequence	Primers used to make probe	Hybridization temperature	Washing temperature	Exposure time
U (Universal)	nt 915-1101 of D109D	Usl-3 and Usl-2	50°C	50°C	2 hours
a	nt 417-760 of D109D	Fsl-1 and Rsl-1	65°C	65°C	2 hours
f	nt 116-364 of D109E	D109E-2 and D109E-3	55°C	60°C	Overnight

D109 (1/20) DNA as template was initially performed and the major product was gel purified with the Qiaex II kit following the manufacturer's protocol for agarose gel extraction and used as template in the Usl-3/Usl-2 PCR. The major product was gel purified and quantitated. For the other probes, the PCR was performed using dilute (1/20) D109 DNA as template and the major fragment was gel purified and quantitated. Probes were labelled with the Invitrogen random primer labelling kit (Cat. no. 18187-013) according to the manufacturer's directions at room temperature for 2 hrs, stopped, and then stored at 4°C until use.

The gel was prepared for DNA transfer to nitrocellulose as follows: 45 min washing in denaturing solution (0.5M NaOH with 1.5M NaCl), washing briefly several times in sterile MilliQ H_2O, then washing in neutralizing solution (1M Tris-HCl and 1.5M NaCl [pH 7.5]) for 30 min and then 15 min more in fresh neutralization buffer. A nitrocellulose membrane (Nitropure supported nitrocellulose, 45 micron [Osmotics Inc, Livermore, CA; Cat. no. WP4HY00010]) was placed on top of the gel and the capillary transfer was prepared according to Sambrook et al. [33] using 10X SSC (1.5M NaCl, 0.15M NaCitrate) and left overnight. The membrane was baked under vacuum for 2 hrs at 70-80°C.

Hybridizations were performed as follows: membranes were prehybridized at the hybridization temperature in a hybridization oven for 1.5 to 2 hours with 8 to 10 mL of hybridization buffer (6X SSPE, pH 7.4 [0.9M NaCl, 0.06M Na_3HPO_4, 0.06M EDTA], 0.5% SDS [w/v], 5X Denhardt's [0.1% Ficoll 400, 0.1% Polyvinylpyrrolidone, 0.1% BSA fraction V]) with 100 µg/mL calf thymus DNA (Sigma, Cat. no. D8661) that was denatured by boiling for 5 min. The appropriate radiolabelled probe (Table 3.2) was

added to the hybridization tube after boiling for 5 min and the membrane was hybridized overnight. The following day the membrane was washed in high salt buffer (2X SSC [0.3M NaCl, 0.03M $Na_3C_6H_8O_7$], 0.1% SDS [w/v]) for 3 to 7 minutes at room temperature, followed by washing in low salt buffer (0.1X SSC [0.15M NaCl, 0.0015M $Na_3C_6H_8O_7$], 0.1% SDS) at the temperatures listed in Table 3.2. The membrane was sealed in a plastic bag and exposed to a Fuji phosphoimaging plate (that had been previously erased for 15 minutes under bright light) for the times indicated in Table 3.2. The image was captured from the plate by exposing in a Fuji (Stamford, CT) BAS-1800 phosphoimager. The membrane was stripped by heating to 90°C in 2X SSC (0.3M NaCl, 0.03M $Na_3C_6H_8O_7$) for 5 min, then placing in ice cold sterile MilliQ H_2O for 5 min. The effectiveness of the stripping procedure was checked by exposure of the stripped membrane to an erased phosphoimaging plate for 2 hrs, as above.

3.3 Results

3.3.1. Genetic characterization of the *L. gallinarum* isolates using PFGE, RAPD, and plasmid profiles

The investigation of the microbial ecology of the chicken crop over the life of the broilers indicated that *L. gallinarum* and *L. crispatus* were present throughout the 6-week production period (Chapter 2 Figs. 2.5 & 2.6, Table 2.8). During the preliminary experiment ("1st experiment", Chapter 2 Table 2.5), 26 *L. gallinarum* isolates were identified. During the crop ecology experiment ("2nd experiment", Chapter 2 Table 2.6), 18 *L. gallinarum* isolates were identified from the crops of chickens and characterized. Two isolates, D109 and 195#2, were confirmed as *L. gallinarum* by partial 16S rDNA sequencing (D109 gave 99.6% identity to nt 160 - 649 of GenBank entry LGA417737 and D195#2 gave 98.6% identity to nt 144 - 649 of GenBank entry LGA417737).

In preparing cultures for further genetic characterization, glycerol stocks of the isolates were streaked to dilution on MRS and purified as described in section 3.2.1.1. Multiple colony types were obtained from the glycerol stocks of seven *L. gallinarum* isolates from the first experiment as follows (Table 3.3): two different colony types were purified from isolates D44, D149, D197, D260, D75 and D80; and three colony types were purified from D195. DNA was isolated from the purified cultures and used as template in the *Mse*I-ARDRA to confirm the species designation (Chapter 2 Fig. 2.8). The identity of all purified cultures was confirmed as *L. gallinarum,* except for D75#2 and D80#1 which were identified as *L. crispatus*.

3.3.1.1. Groupings based on PFGE, RAPD & plasmids

*Sma*I PFGE was performed on all 44 *L. gallinarum* isolates with the analyses combining the results obtained from several different gels shown in Fig. 3.2. In some

Table 3.3. Summary of *L. gallinarum* isolates obtained from chicken crop

PFGE group (strain)	Isolates	Chicken no.	Chicken age (days)	Experiment no.	Growth in MRS broth	Colony morphology[2]
1	D41	1	2	1	Good	W, O, SM, S, R
	D109	1436	5	1	Poor	W, O, SM, S, R
	D148	1441	14	1	Good	T, SM, S, R
2	D45	1	2	1	Good	W, O, VS, S, R
	D71	3	2	1	Good	W, O, L, F, R
	D73	3	2	1	Good	W, O, L, F, R
	D75#1	3	2	1	Good	T, S, B
	D79	3	2	1	Good	W, O, L, F, R
	D80#2	3	2	1	Good	W, O, M, R, B
	D108	1436	5	1	Good	W, O, L, F
3a	D149#1	1441	14	1	Good	W, O, F, R
	D149#2	1441	14	1	Poor	W, O, S, R, B
	D195#2	1464 contents only	14	1	Good	ST, R, S
	D195#3	1464 contents only	14	1	Good	ST, R, B
	D197#1	1464 contents only	14	1	Good	W, O, F, B
	D197#2	1464 contents only	14	1	Good	W, O, I
3b	D195#1	1464 contents only	14	1	Poor	ST, R, S
4a	D42	1	2	1	Good	W, O, S, F
	D47	1	2	1	Good	W, O, M, R, B
4b	D46	1	2	1	Good	O, VS, F
5	D44#1	1	2	1	Poor	W, O, F, B
	D44#2	1	2	1	Good	T, S, B
6	D260#1	1461	11	1	Good	W, O, F, B
	D260#2	1461	11	1	Good	W, O, I
7	15-5	15	1	2	Good	W, O, SM, S, R
	15-8	15	1	2	Good	W, O, S, F
8	28-1	28	3	2	Good	W, O, L, F
	28-6	28	3	2	Good	W, O, L, F
	28-7	28	3	2	Good	W, O, L, F

[1]N/A - not applicable

[2]W - white, O - opaque, T - translucent, ST - semi-translucent, R - round, F - fuzzy, L - lumpy, SM - smooth, L - large, M - medium, S - small, VS - very small, B - Bulls-eye, I - irregular

[T]Type strain

Table 3.3. Continued

PFGE group (strain)	Isolates	Chicken no.	Chicken age (days)	Experiment no.	Growth in MRS broth	Colony morphology[2]
9	28-3	28	3	2	Good	T, L, F
	28-4	28	3	2	Good	T, L, F
	28-9	28	3	2	Poor	T, M, F, B
	31-2	31	7	2	Good	W, O, S, L, B
	31-5	31	7	2	Good	W, O, S, L
	31-7	31	7	2	Good	W, O, S, L, B
	31-9	31	7	2	Good	W, O, SM, S, R
10	D255	1461	11	1	Good	T, SM, M, R
11	D256	1461	11	1	Good	W, O, SM, S, R
12	15-4	15	1	2	Good	W, O, S, F
13	15-6	15	1	2	Good	ST, S, F, B
14	15-9	15	1	2	Good	W, O, SM, VS, R
15	26-3	26	3	2	Good	W, O, M, F, B
16	31-4	31	7	2	Poor	W, O, L, F
17	31-6	31	7	2	Good	W, O, SM, M, R
18	ATCC 33199^T	[15]	N/A[1]	N/A	Good	W, O, S, R, B

[1]N/A - not applicable

[2]W - white, O - opaque, T - translucent, ST - semi-translucent, R - round, F - fuzzy, L - lumpy, SM - smooth, L - large, M - medium, S - small, VS - very small, B - Bulls-eye, I - irregular

^{T}Type strain

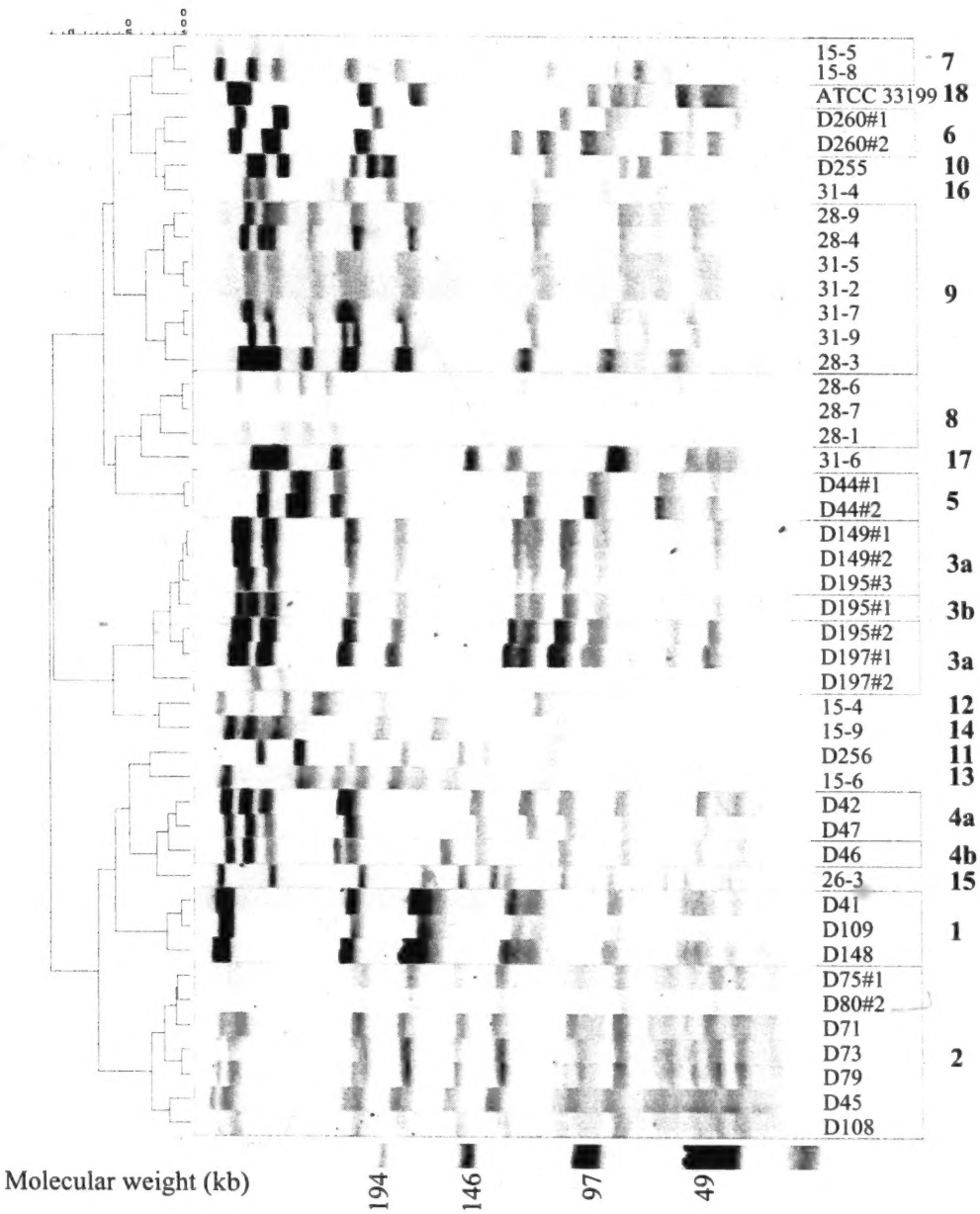

Fig. 3.2. Comparison of *Sma*I-PFGE profiles of selected *L. gallinarum* isolates digested with *Sma*I and run in 1% agarose. Bold numbers on the right indicate PFGE groups (Table 3.3).

circumstances, identical patterns were slightly shifted due to variations in amount of cells in the agarose plugs, plug slice thickness or slight migration of the gel slices into the molten agarose before solidification. Nevertheless, the algorithm was able to detect identical patterns and group them together (i.e. compare the worst case scenario of D260#1 and D260#2 in Fig. 3.2). The patterns observed were significantly different between PFGE groups and highly consistent within PFGE groups, making both visual and computer analysis easier than with RAPD (see below). The results from PFGE groupings are summarized in Table 3.3. PFGE revealed that 17 strains (PFGE groups 1-17) are represented among the 44 isolates. Slight variations in the PFGE patterns were noted in PFGE groups 3 and 4, designated by subgroupings "a" and "b". When comparing PFGE patterns of groups 3a and 3b, D195#1 (PFGE group 3b) consistently differed only slightly in the migration of a very high molecular weight band. Further, the patterns of D46 (PFGE group 4b) was similar to D42 and D47 (PFGE group 4a) except that one band (~97 kb) was absent and two additional bands of ~140 kb and ~200 kb were present. No isolates were similar to ATCC 33199, the *L. gallinarum* type strain.

*Apa*I is another enzyme commonly used in PFGE typing of lactobacilli. This enzyme was used to confirm the groupings of the largest *Sma*I groups (PFGE groups 2, 3 and 9). The patterns obtained from groups 2 and 9 agreed with the *Sma*I data, but the enzyme did not digest the DNA for PFGE group 3 (data not shown). As *Sma*I and *Apa*I digests gave the same results for the largest PFGE groups and did not digest the DNA of some PFGE groups, *Apa*I digests were not performed on the remaining isolates.

The origin of the isolates representing the different PFGE groups was investigated (Table 3.3). Isolates from the same crop were more likely to be the same strain (PFGE

group), although several strains were detected in different crops (i.e. PFGE groups 1, 2, 3 and 9). For example, isolates belonging to PFGE group 8 were only obtained from bird 28 and were not detected in other birds to date. Further, some strains appear to be observed in the crops of birds of different ages: in PFGE group 1, the three isolates were observed in three different birds of 2, 5, and 14 days of age; the isolates in group 2 were observed in birds of 2 and 5 days of age; and the isolates of PFGE group 9 are observed in chickens aged 3 and 7 days. Interestingly, there were no identical strains detected from both chicken experiments. PFGE analysis indicates that a given chicken crop is likely to contain multiple strains of *L. gallinarum* (Table 3.3). For example, chicken crop 1 contained isolates from PFGE groups 1, 2, 4, and 5 and chicken crop 15 contained isolates from PFGE groups 7,12, 13 and 14). Crops obtained from chickens 1 or 2 days old (i.e. crop 1 and 15) had the most diversity (four strains), while crops obtained from older chickens (e.g. crops 1441, 1461, 1464, 31) had the least diversity (three or fewer strains). However, given the limited number of *L. gallinarum* isolates obtained per crop, it is difficult to determine an age-dependent relationship with species diversity.

RAPD was also tested as a quick and simple method of investigating the relationships among selected *L. gallinarum* isolates. DNA from the isolates was used as template in PCR containing OPA-02 or OPA-18 primers [32], and the resulting banding patterns analysed using Bionumerics (Fig. 3.3). Using patterns from both primers, RAPD was able to distinguish several major groups of *L. gallinarum* strains. OPA-02 separated 23 isolates into 8 clusters, and OPA-18 produced 7 slightly different groupings. These groupings were quite similar to those for PFGE, although there are some inconsistencies between the RAPD groupings and those determined by PFGE. For example, with

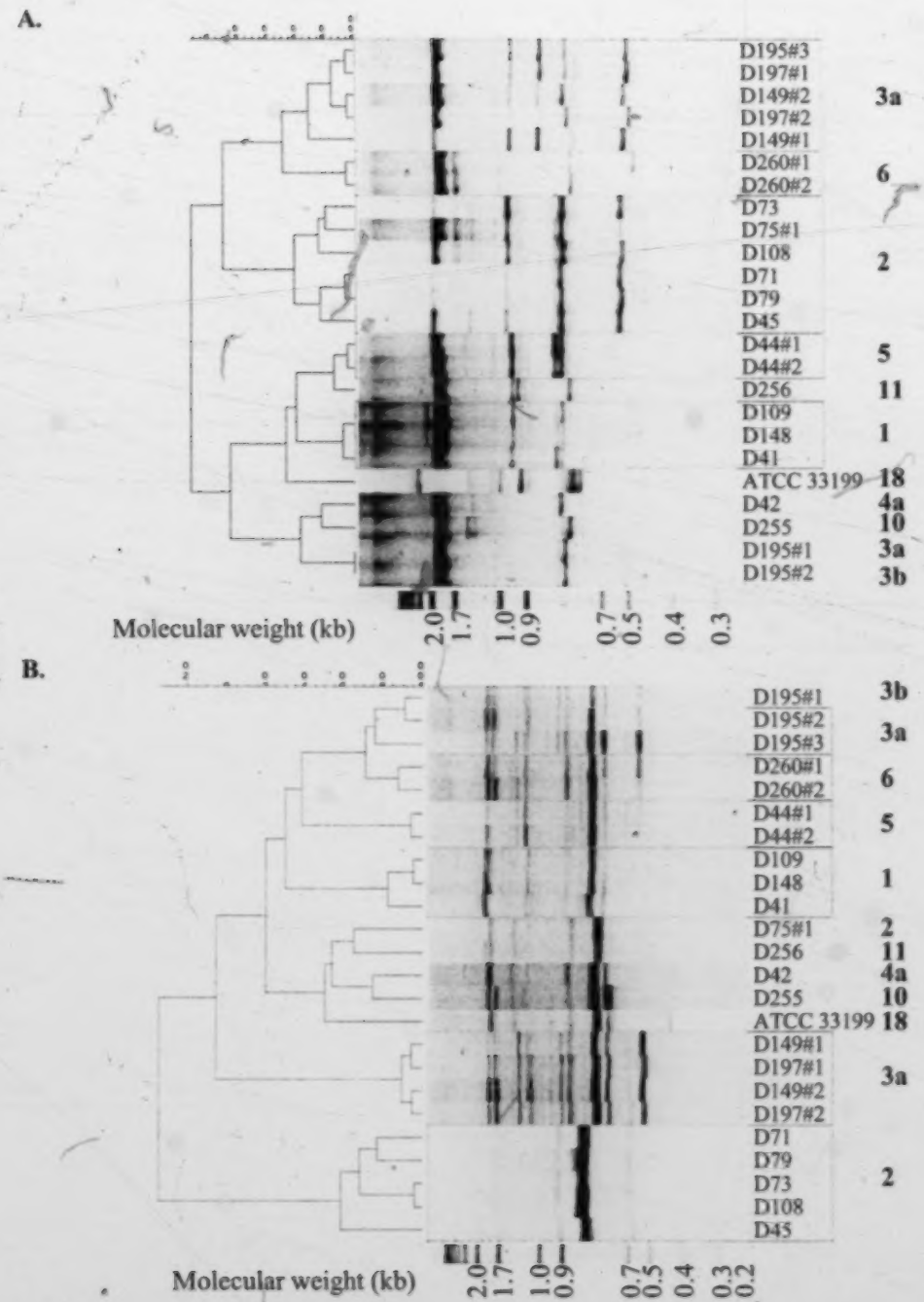

Fig. 3.3. Comparison of RAPD profiles of selected *L. gallinarum* isolates amplified with **A.** OPA-02 and **B.** OPA-18. Bold numbers represent PFGE groups as determined in Fig. 3.2.

OPA-02, D42 (PFGE group 4a) and D256 (PFGE group 11) were clustered similar to D195#1 and D195#2 (PFGE groups 3a and b). Further, with OPA-18 D75#1 (PFGE group 2) is clustered closer to D256 (PFGE group 11) than with the remainder of PFGE group 2. The simplicity of patterns (as seen with PFGE group 2 using OPA-18) or complexity of patterns (as seen with PFGE group 3 using OPA-18) likely contribute to the inability to definitively distinguish the *L. gallinarum* strains. Further, gel-to-gel comparisons appears to be difficult with RAPD patterns: for OPA-18, D75#1 was run on a separate gel from the remainder of PFGE group 2, likely explaining the Bionumerics analysis. Therefore, PFGE and RAPD data used to characterize *L. gallinarum* strains often agreed, but several circumstances of disagreement between PFGE and RAPD were observed.

Plasmid profiles from the 44 *L. gallinarum* isolates are shown in Fig. 3.4. All the isolates contained at least one plasmid, and in some instances, multiple bands in the profile (e.g. isolates from chicken 28), which could either, represent multimers of the plasmids, different forms of the plasmids, and/or multiple plasmids. The majority of isolates have plasmid profiles similar to those of other members of their PFGE group. Several noticeable differences are seen with D256 (PFGE group 11), which has a profile identical to that of PFGE group 2. Isolate 15-4 has a similar plasmid profile to 15-8; yet 15-5, which belongs to the same PFGE group (8) was different. Within PFGE group 9, the isolates from chicken 28 (28-3, 28-4, 28-9) have the same profile, while those isolates from chicken 31 (31-2, 31-5, 31-7, 31-9) have a different profile. Further, PFGE groups 5 and 6 have very similar profiles despite being isolated from different chickens of different ages. Therefore, while isolates from the same PFGE group often had the same

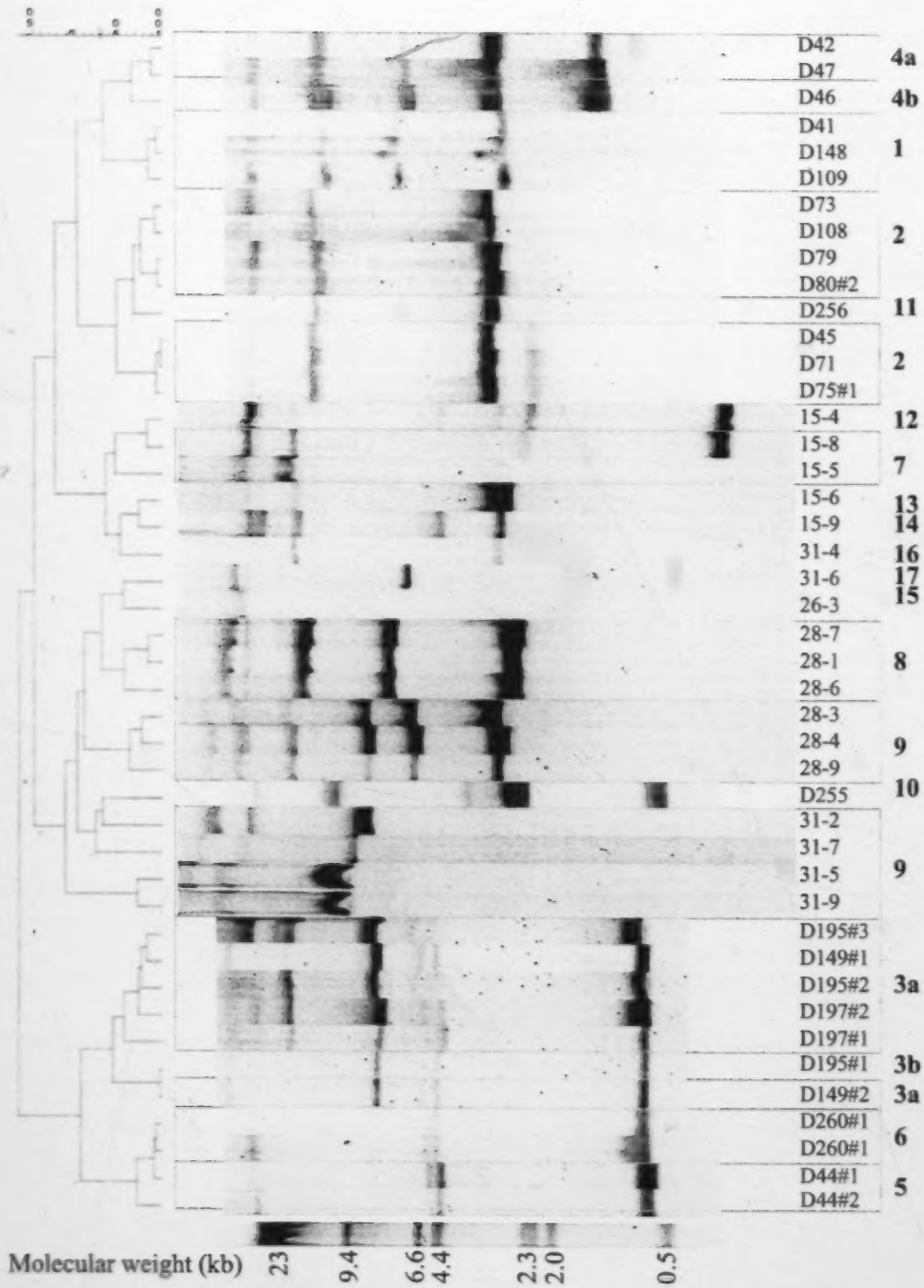

Fig. 3.4. Comparison of plasmid profiles of selected *L. gallinarum* isolates. Bold numbers represent PFGE groups as determined in Fig. 3.2.

plasmid profiles, isolates from the same chicken crop were also likely to have the same profile. Therefore, *L. gallinarum* plasmid profiles are not a reliable method for strain identification.

3.3.2. SDS-PAGE profiles of *L. gallinarum* isolates

To determine if *L. gallinarum* crop isolates produced S-layer proteins, extracellular proteins were extracted and subjected to SDS-PAGE. The protein profiles of all isolates contained faint bands as well as a noticeable protein between 43-52 kDa (Figs. 3.5 & 3.6), which is consistent with the size of the S-proteins produced by other species of the group A *acidophilus*. A range of ~9 kDa (43-52 kDa) was observed for S-protein sizes (Fig. 3.5), and representative isolates expressing S-proteins of different sizes were used to make the S-protein marker (SPM), which was used as shown in Fig. 3.6. The amount of the S-protein that was visually evident on the gel varied somewhat: strains that grew well had a significant protein band whereas strains that grew poorly had a fainter band. In effect, the amount of S-protein that was evident on SDS-PAGE correlated to growth in MRS broth (data not shown, Table 3.3).

To investigate whether isolates in the same PFGE grouping produced similar S-proteins, the SDS-PAGE profile of various strains was compared (Fig. 3.6). Indeed, a correlation between strain (as determined by PFGE group, Table 3.3) and S-protein size (produced in MRS media under anaerobic conditions) was observed (Fig. 3.6). Isolates belonging to the same PFGE group produced S-proteins of the same size as shown in Fig. 3.6. However, isolates belonging to different PFGE groups were also observed to produce S-proteins of the same or different size. For example, isolates belonging to groups 1 and 8 in Fig. 3.6D produce very similar sized S-proteins. Nevertheless, isolates from PFGE

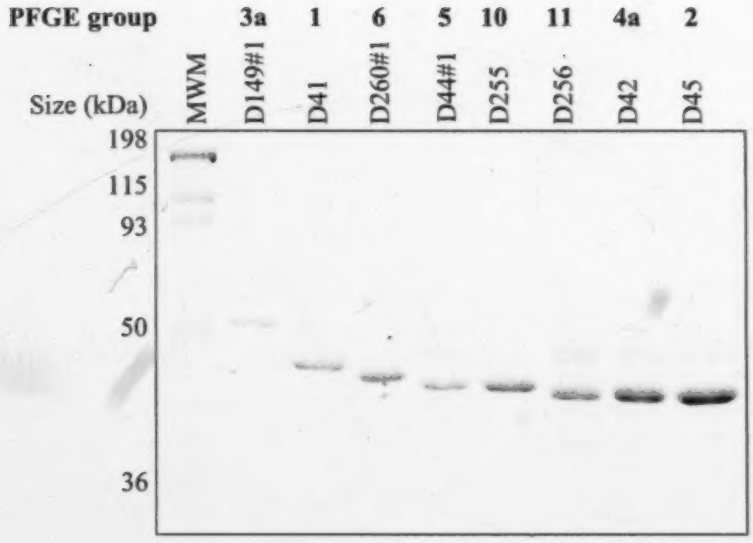

Fig. 3.5. SDS-15% PAGE of extracellular protein extracts of *L. gallinarum*. MWM - molecular weight marker. Bold numbers represent PFGE groups as determined in Fig. 3.2.

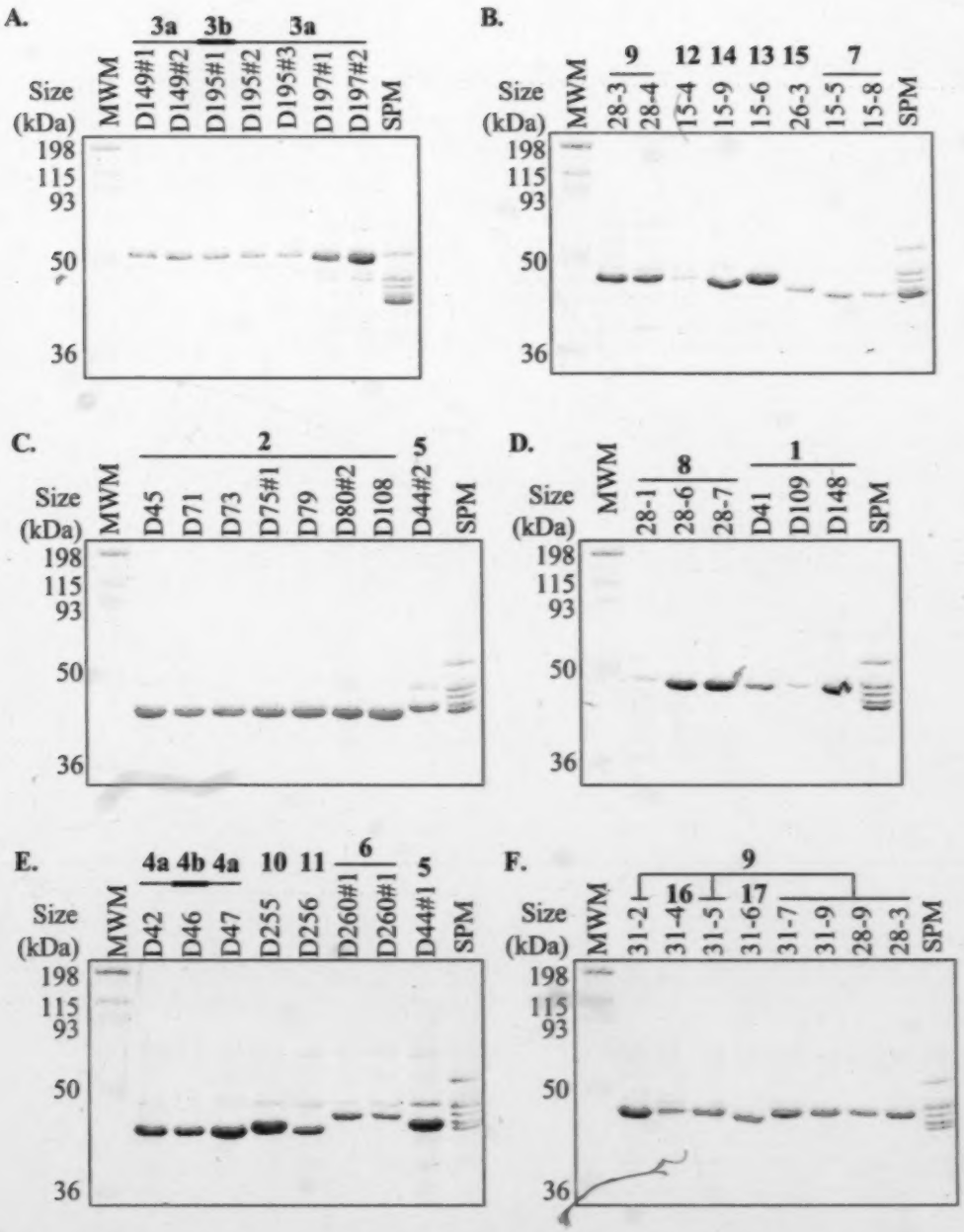

Fig. 3.6. Extracellular protein profiles extracted from all characterized strains of *L. gallinarum* and run on SDS-15%PAGE. Lane numbers are strain identification numbers. Bold numbers represent PFGE groupings as determined in Fig. 3.2. Thicker lines represent closely related but slightly different strains. MWM - molecular weight marker. SPM (S-protein marker) derived from the S-proteins shown in Fig. 3.5 and consists of (in descending order): D149#1, D41, D44#1, D260#1, D45.

group 3a and 3b produced S-proteins considerably larger than all other isolates tested. Confirmation of the correlation between S-protein molecular weight and strain was obtained by analysis of the samples run in different gels represented in Fig. 3.6 with Bionumerics (data not shown). In general, the extracellular protein profiles represented in Fig, 3.6 are also in agreement among isolates of the same strain, although not exclusive to that strain.

3.3.3. Cloning and Sequencing of S-protein genes

PCR products were amplified from the GAA type strains by primers Usl-1 and Usl-2, confirming the ability of the primers to detect S-protein genes. The *L. acidophilus* group A type strains tested positive for the S-protein gene confirming that the oligonucleotides are not species-specific (Fig. 3.7), as expected, since the primers Usl-1 and Usl-2 (Table 3.1, Fig. 3.1) were designed based on the homologous gene sequences encoding the conserved N- and C-terminal domains in *L. acidophilus* and *L. crispatus* S-protein genes (Chapter 1 Fig. 1.3). The negative control, containing only sterile MilliQ H_2O, did not have any products. The *L. gallinarum* isolates were screened for the presence of S-protein genes. Analysis of the PCR products on agarose gel revealed that all *L. gallinarum* isolates tested had at least one PCR product of the expected size (~1.2 kb), although other fainter PCR products were observed in some strains (Fig. 3.7 and data not shown).

Usl-1/Usl-2 PCR products from *L. gallinarum* isolates were cloned into *E. coli* for sequencing. A total of eight clones were selected for sequencing, and these were designated by the name of the original isolate followed by letters in alphabetical order.

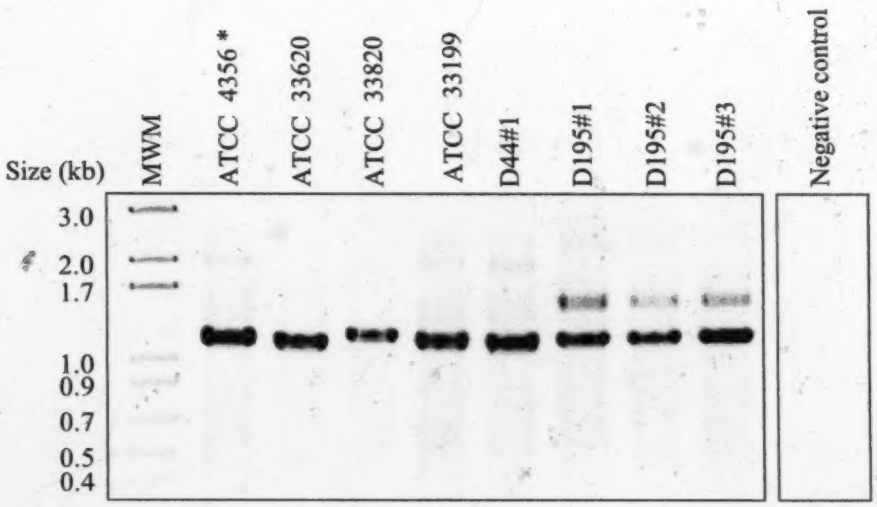

Fig. 3.7. Agarose gel electrophoresis (1%) of representative Usl-1/Usl-2 PCR reactions containing template DNA from the strains indicated. "*" indicates positive controls. MWM - molecular weight marker.

When S-protein gene fragments were cloned in *E. coli* JM109 and sequenced, several unique gene types were obtained (Table 3.4). Gene type a, initially cloned and sequenced from *L. gallinarum* D109, was also cloned from isolates D108, D255, D256 and D260#1. Slight heterogeneity was observed for gene type a sequences (Appendix Fig. A.2). Gene type b, originally cloned from D195#2 was also cloned from isolates D149#1, D195#1, D197#1, D197#2 and ATCC 33199, with very high sequence identity (data not shown). Gene types c through g were only cloned from one isolate each (Table 3.4).

Complete variable region sequences were obtained for the representative clones selected for gene types a, b, c, and e. Due to poor quality sequence or the presence of multiple PCR products, complete variable region sequences were not obtained for clones d, f, g and h. The sequences of the complete variable region sequences are compared in Fig. 3.8A. Gene type c is very dissimilar to the other sequences and appears to have several regions of "extra" DNA, as seen by the gaps in Figs. 3.8 and 3.9. The 5' end of all 8 unique sequences from representative clones are compared in Fig. 3.8D. Again, gene type c is very dissimilar to the other sequences in Fig. 3.8D. Predicted proteins for the completely sequenced variable regions are compared with each other in Fig. 3.9 and to the published *L. acidophilus* and *L. crispatus* proteins in Fig. 3.10. Several regions of homology exist among the proteins, as indicated by blue boxes in Fig. 3.10A. Several areas of unique sequence can be observed in S-protein types a and b, as indicated by green boxes in Fig. 3.10A. S-protein types a and b are closely related to type e with 67% and 61% identity, respectively, however S-protein type e is more closely related to SlpnB with 82% identity (Fig. 3.10B & C).

Table 3.4. Cloning and sequencing of S-protein gene types from *L. gallinarum* crop isolates.

Gene type	Representative clone	Cloned from other strains	Sequence quality
a	D109D	D108, D255, D256, D260#1	Good
b	D195#2F	D149#1, D195#1, D197#1, ATCC 33199	Good
c	D195#2C	N/A	Poor in areas
d	D255B	N/A	Poor in areas
e	D256A	N/A	Good
f	D109E	N/A	Poor in areas
g	D44#2B	N/A	Good
h	D42C	N/A	Good

A.

```
Clone D109D (a)     TGCTTTATTAGCTGTTGCTCCTGTTGCTGCTTCTGCTGTAAGCACC---G 47
Clone D1952#F (b)   T---TTATTAGCTGTTGCTCCTGTTGCTGCTTCTGCTGTAATCACT---G 44
Clone D195#2C (c)   TGCTTTATTAGCTGTTGCTCCTGTTGCTGCTTCTGGCGTTGTTGCTCCAG 50
Clone D256A (e)     TGCTTTATTAGCTGTTGCTCCAGTTGCTGCTTCCGCTGTATCTACT---G 47
                    *   ****************** ********** *  **         *

Clone D109D (a)     TTAACGCTGCTAGTGTTACTTCCGCTAC-----TCAA----TTAGGTAAG 88
Clone D1952#F (b)   TTAACGCTGCTGCCGTTACTACTGCTAC-----TCAA----CTTGGTGGT 85
Clone D195#2C (c)   CTTCAGTTGTTTCAGCTGCTACTACTGCAGATGTTAAGGTACCAGGTGTT 100
Clone D256A (e)     TTTCAGCTGATCCTGTTCAAACTGCTAC-----TCAA----CTTGGTACT 88
                     *   * ** *   * *    *  ** *     * **       ***

Clone D109D (a)     GTACCT--ACTTTA----GCTAATGGTGA---TGCTGTAAA--TG---TT 124
Clone D1952#F (b)   GTAAAA--TTACCA----ACTTCAAATGC---TGCTGTAAA--TG---TT 121
Clone D195#2C (c)   ATTACTTTACACCGTGGCACCAACAGCCAATTTACTCTTAGCTTGAATTC 150
Clone D256A (e)     GTACCT--GCTTTA----TCAAACGGTGA---CACTGTTAA--CG---TT 124
                     *                *               ** ***        *

Clone D109D (a)     AAGCCAAA----TGTTTCATTA-AACA--------C---AGTACATG--- 155
Clone D1952#F (b)   AAGCCAAA----CGTTTCATTA-AATA--------CTGCTGTAGGTA--- 155
Clone D195#2C (c)   AACTCAAGAGGCTAGTTTATTACAACAACTTAAAGCTCCAGCTGGCGCAA 200
Clone D256A (e)     AAGCCAAA----TGTTTCATTA-AACA--------CTTCAGCTTATG--- 158
                    **  ***        ** **** ** *        *    *

Clone D109D (a)     -----------GTTCA-ATTAAG---------GCAGCTATTTCT----GT 180
Clone D1952#F (b)   -----------ACAGC-GTTAAG---------GCTTCAATTTCA----GT 180
Clone D195#2C (c)   AGATCAGCAATGTAGTTGTTAAGCAAGTTACTGCTACTAACCCAAATGGT 250
Clone D256A (e)     -----------AAGGT-GTTAAG---------GCAAACATCTCA----GT 183
                               *****  *      **    *   *    **

Clone D109D (a)     A---TCATTTGACGCT--ACTTTTAACG---------------------- 203
Clone D1952#F (b)   A---TCATTTACTGCT--ACTATTGACG---------------------- 203
Clone D195#2C (c)   GAATTTAACCAATACTTTACTTTTGATGTCGTAAATGAAAAGGGTGAAGT 300
Clone D256A (e)     A---TCATTCTCAGCT--GCTGTTAATG---------------------- 206
                        * *        **   ** ** * *

Clone D109D (a)     --------GTACTACTGCTACCTCAAACTTTAAGCCTG--GATACTCAAA 243
Clone D1952#F (b)   --------GTACTACTGCTACTGCTAACTTAGATCCTA--AGTCTACTGA 243
Clone D195#2C (c)   TCTTAAGAGTGCTACTGGTATGACTAATGCTAAGTTTGCTAGTCAACAAG 350
Clone D256A (e)     --------GTACTACCGCTATTTCAAACTTTAAGCCAG--GTGTTTCAGA 246
                            ** **** * **   * **     *            *

Clone D109D (a)     C-ATCCAAC---TTT------TCCACGGTAGCAAGGAAATT--------- 274
Clone D1952#F (b)   A-GTAAGTC---TCTACAAGGGTACTGTTTCAGATGCAAATAAA------ 283
Clone D195#2C (c)   GTACTCCATACTTCTACAATATTGCAGATAACACTGTTATCAACAATGGT 400
Clone D256A (e)     A-ATTTCAC---TTTGGAAAGTTGAAAAGGACAAGGTTACTCAC------ 286
                           * *

Clone D109D (a)     ---------ACT------AAT-TTACAA---------------------- 286
Clone D1952#F (b)   ------GTTACC------GAC-TTACAA---------------------- 298
Clone D195#2C (c)   AGCACTGTAACTATCAATGATATTGCAAACGGCTTCACTCCATCTTCACT 450
Clone D256A (e)     ------GTAACT------GAT-TTACAA---------------------- 301
                          **          *  ** ***
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Fig. 3.8. Comparison of DNA sequences of *L. gallinarum* S-protein genes. **A.** ClustalV alignment of DNA from 4 complete variable region sequences. "*" indicates identical nucleotide in all sequences. **B.** Phylogenetic tree based on ClustalV DNA sequence comparisons. **C.** Percent identity of DNA sequences. **D.** ClustalW alignment of 8 5' sequences from partial and complete S-protein gene variable regions. "*" indicates identical nucleotide in all sequences. **E.** Phylogenetic tree based on ClustalW DNA sequence comparisons. **F.** Percent identity of DNA sequences.

A.

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Clone D109D (a)     ---GATGTTCATTACC-------------------TTACTGCAGGTTCAA 314
Clone D1952#F (b)   ---GACGTAACTTCAG-------------------CTGATGCTGGTACAG 326
Clone D195#2C (c)   TTTGAAGGAAATTAACGATAAGTATGCATGGAAGACTAGTGATGCTTCAG 500
Clone D256A (e)     ---AAAGTAACTTCAT-------------------CAAACGCTGGTGCTA 329
                       * *    **                          *  * *

Clone D109D (a)     CTTACCATGTTGTAATGAGCCATGTTGGTTTAAACTTTG------------ 353
Clone D1952#F (b)   AATATAAAGTTGGTATGAACAACGTTTCTTTAAACTTTG------------ 365
Clone D195#2C (c)   GCTCAAAGGCTGAAATGACTACTACTGCTTCTGATGTTGAAAGTCAATTA 550
Clone D256A (e)     CTTACCAAGTTAAAATGACTAATGTTGGTTTGAACTTTG------------ 368
                      *   * * *   ****        *  **   *  ***

Clone D109D (a)     ---GTTCA-----CAAAACGCAAACAAGGAAATCACTTTAACTATGC--C 393
Clone D1952#F (b)   ---GTTCA-----CAAAATGCTAACAAGAAAGTTACTTTGACTTTCC--C 405
Clone D195#2C (c)   GTTGCTCAAGGCTTGAAACGTGCTTCAAACGGTGACTTTGATTACCCAGC 600
Clone D256A (e)     ---GCTCA-----CAAAATGCTAACAAGAAGATTACTTTAACTTTCC--C 408
                       * ***       ***         * *   * ***** * *  **  *

Clone D109D (a)     TGAAGGCGATTTCTTCCAACT---TGCA---TCAAACAATACTGTTACAA 437
Clone D1952#F (b)   ---AGATAACGGATCACACTT---TGGC---TACAATGGTAAAGATGGAC 446
Clone D195#2C (c)   AAACGGCTTCAACCTTAAGTTGAGTGCTAAGTCAGAAAATGGTAATACTG 650
Clone D256A (e)     AGAAGGTGATGGCTTTAAGCT---TGCT---TCAAACAACTCATTTACTA 452
                       *           * *  **    *  *         *

Clone D109D (a)     ACTCAAGAACTATTAAGCTTGACCAAAACGGTA-CTGTAAC---------- 477
Clone D1952#F (b)   GTTCACAAGAAGTACAACTTAACAAAGATGGTG-TTGTAAC---------- 486
Clone D195#2C (c)   CTTCAATTACTGTTAGAATTAACGCAGTTGTTAACTACAATGCTCCTGCA 700
Clone D256A (e)     ATTCAAGAACCATTCAACTTGACAAGAATGGTA-CTGTAAC---------- 492
                      ***       *     ** **     * *   *

Clone D109D (a)     CTTGAACCAAGTTGC------TATCA-A-------CGTAACTGCTAAGG- 512
Clone D1952#F (b)   TTTATCATACGTAAT------C-TTCCC-------TGTAACTGCCAAGA- 521
Clone D195#2C (c)   TTCGTAGTAGATAATACTGTTTACTTCAATAACAATGTAACTGTAAATGG 750
Clone D256A (e)     TTTAAATGAAGTTG-------TATTACA-------CGTAACTGCTAAGG- 507
                     *                                *******  **

Clone D109D (a)     ----ACTTT-GCTAACCCAGC--------------------------TG 530
Clone D1952#F (b)   ----ACTTC-GCTAACCCTGA--------------------------AA 539
Clone D195#2C (c)   TACTACTTCAGGTGACTTAGTAATTAGAGATACTAAAGATACTAAGGTTA 800
Clone D256A (e)     ----ACTTT-GCTAACCCAGC--------------------------TG 545
                        ****  * * **

Clone D109D (a)     TTGT---TGCTTGGTACGACA------GAGAAACTAACG---------TT 562
Clone D1952#F (b)   TTGT---TAGTTGGTTCAATG------TTACTACTGGTGC---ACCTGTA 577
Clone D195#2C (c)   TTGTAAATGATAAGCTTAACGAAAAAGCTGTTACTGATGCTGTAACTGTT 850
Clone D256A (e)     TTGT--TAACTGGCTTAACG------CTACTACTAATGC---AGTTGTT 583
                    ****   *     *                ***   *         *

Clone D109D (a)     AACGTA------------ACTAGCGGT-A--ACATTAC---------TTT 588
Clone D1952#F (b)   ACTTCA------------GCAAGCATTCA--ATTATAC-GCT---GGTTC 609
Clone D195#2C (c)   TACGTACAAAGCGCATACGGCAACAGTTATGACAAGACTGCTAAGGAATT 900
Clone D256A (e)     AACTCA------------GGTAACGTTGA--ACTTTAC-GCT---GGCTC 615
                          *                 *  * *  *    **         *

Clone D109D (a)     AGATG----------------------CTGGTAAGATGAACGTTG-CTCAA 616
Clone D1952#F (b)   AAACG----------------------CAGGTAAGATGAACGTTG-CTCAA 637
Clone D195#2C (c)   AAGTGAACACCAACAAAAGATCAACCCAAGCAACATCAAGATTGACTCAA 950
Clone D256A (e)     AGATG----------------------CTGGTAAGATGAACGTTA-ACCAA 643
                    *   *                     *  * ** ** **  **    ***

Clone D109D (a)     TTTGTAGCTGCTGCAGAA--AACA-A--GTATGTTGCACGCAACAATGCA 661
Clone D1952#F (b)   GTTATTTCAGCTGTTAGCCCATTAGA--AAACAAGGGTAACGGCTACGTT 685
Clone D195#2C (c)   GTGCTGTTAACGGTAAGGTTGCAGGTATGTACCCTGTAACTATTACTGCT 1000
Clone D256A (e)     GTTATTGCAGCTGTTCGT--GCTA-A--GTACTATGCAACTAACTTTGGT 688
                     *  *    *                    *    *         *
```

A.

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Clone D109D (a)     GGTGACAAGCACAACAGTCAATCAAGT---ACTATTTCAT---ACAGCAA 705
Clone D1952#F (b)   GCAATGCAATATGGTAACAAGCTTGCTTTCGACACTACAGCTAACATTAA 735
Clone D195#2C (c)   ACTAACCCAGCTGGTTTTACCTCAAAGCTTGTTGTTT-ATGTAATCGTAA 1049
Clone D256A (e)     ACT--TTAACA-AACAAGGAATCAAGT---GACATCAAGT---ACACTGA 729
                                                              *    *

Clone D109D (a)     CAA--CAT--CAAAGAAGCTTTAA------------AGGCTATGAACATT 739
Clone D1952#F (b)   CAA--CCT--TAAATCAGCTCTTA------------AGGCTATGAACATT 769
Clone D195#2C (c)   TGGGTCCTACTGAAAAGGTTCCAGCTCAATACGTACATGTTGACAATGCT 1099
Clone D256A (e)     CAA--CCT--TGTTGAAGCTTTAA------------AGGCTGCTGGTGTT 763
                          ***          * *                 * * *       *

Clone D109D (a)     GATATT---GATGCTAATGGTTGGTTTGT---TG------CTCC---TAA 774
Clone D1952#F (b)   GATGTT---GACGCTAACGGTTGGTTTGT---TG------CACC---TAA 804
Clone D195#2C (c)   AAGGTTTACAACATCAACGGTAACGTTGTAACTGAAGATTCATCAATTAA 1149
Clone D256A (e)     GAGGTT---AAGG---ATAATTGGTTCGT---TG------CACC---TAA 795
                     *  **    *     *   *    * **   **      * **   ***

Clone D109D (a)     GTCATTTACTT------------TTGAAATGACTGCTTCAGCAAACAACA 812
Clone D1952#F (b)   GTCATTCACTT------------TCAACTTGACTGCAACTTCACCTAAGA 842
Clone D195#2C (c)   GCCACTTGCTAAGGGTGCTGCTGTTTACGCATTTGACTCAGTAACTGTTA 1199
Clone D256A (e)     GTCATTCACTT------------TCAACATGACTGCAACTGCTAACAACA 833
                    * ** *  ***            *  *      **   *          *

Clone D109D (a)     ATGATGCTT----CAGCTAAGTTACCAATTA---CT-----------GTT 844
Clone D1952#F (b)   ACGACGCTA----CTGCAACTTTAGCTGTAA---CT-----------GTT 874
Clone D195#2C (c)   ACGGTGTTAAATACACTGAAATCAACAGTAAGGGCTCAAACAAATGGGTA 1249
Clone D256A (e)     ACGATGCTT----CAAAGACTTTAGCTGTAA---CT---------A--GTT 865
                    * *    *     *    *  * * *  ***   **           **

Clone D109D (a)     ACTGTTCCAAAC------GGTAAGGACGTAACTCTAGCTACTGTTCCAAG 888
Clone D1952#F (b)   AACGTACCAAAC------GGTGTAAACCCTACTCCAGCAACTGTTCCAAG 918
Clone D195#2C (c)   AAGGCTTCAGACTTAACTGCTACTAAGCCAGCTCCAGCTGTAGTTGCAAG 1299
Clone D256A (e)     AACGTACCAAAC------GGTAAGGAAGTAACTCCAGCTACTGTTCCAAG 909
                    *  *   ** **      * *    *     *******    *** ****

Clone D109D (a)     TCAAAGCAAGACTATTATGCACAACGCATACTTCTACGACAAGGACGCTA 938
Clone D1952#F (b)   TCAAAGCAAGACTATTATGCACAACGCATACTTCTACGACAAGGACGCTA 968
Clone D195#2C (c)   CCAAAGCAAGACTATTATGCACAACGCATACTTCTACGACAAGGACGCTA 1349
Clone D256A (e)     TCAACCTAAGACTATTATGCACAACGCATACTTCTACGACAAGGACGCTA  959
                     ***   *********************************************

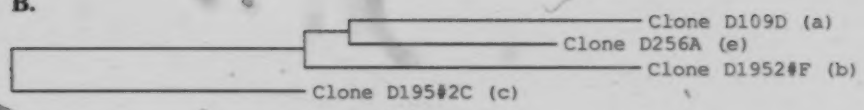
Clone D109D (a)     AGCGTGTTGGTACTGACAAGGTCACCCGTTACAACACTGTAACTGTTGCT 988
Clone D1952#F (b)   AGCGTGTTGGTACTGACAAGGTAACTCGTTACAACACTGTAACTGTTGCT 1018
Clone D195#2C (c)   AGCGTGTTGGTACTGACAAGGTAACTCGTTACAACACTGTAAATGTTTCA 1399
Clone D256A (e)     AGCGTGTAGGTACTGACAAGGTTACCCGTTACAACACTGTTAATGTTTCA 1009
                    ******* ********************************** **** *

Clone D109D (a)     ATGAACACCACTAAGCTTGCTAACGGTATTTCATACTACGAAGTTATCGA 1038
Clone D1952#F (b)   ATGAACACTACTAAGTTCTCAAACGGTATCGAATACTACGAAGTAATCGA 1068
Clone D195#2C (c)   ATGAACAAGACTAAGTTCTCAAACGGTATCGAATACTACGAAGTAATCGA 1449
Clone D256A (e)     ATGAACAAGACTAAGTTCTCAAACGGTATCGAATACTACGAAGTAATCGA 1059
                    *******  ********************   ******************

Clone D109D (a)     AAACGGCAAGGCAACTGGCAAGTACATCAACGCTGACAACATCGACGGTA 1088
Clone D1952#F (b)   AAACGGCAAAGCAACTGGCAAGTACATCAACGCTGACAACATCGACGGTA 1118
Clone D195#2C (c)   AGGCGGTAAGGCAACTGGCAAGTTCATCAACGCTGACAACATCGATGGTA 1499
Clone D256A (e)     AGGCGGTAAGGCAACTGGCAAGTTCATCAACGCAGACAACATTGATGGTA 1109
                    *  *** ** ************** ********* ******** ** ****

Clone D109D (a)     CTAAGCGTACTTT 1101
Clone D1952#F (b)   CTAAGCGTACTTT 1131
Clone D195#2C (c)   CTAAGCGTACTTT 1512
Clone D256A (e)     CTAAGCGTACTTT 1122
                    *************
```

B.



C.

	Clone D109D (a)	Clone D1952#F (b)	Clone D195#2C (c)	Clone D256A (e)
Clone D109D (a)		63	41	67
Clone D1952#F (b)			40	64
Clone D195#2C (c)				41
Clone D256A (e)				

D.

```
Clone D109D (a)   ---TGCTTTAT-TAGCTGTTGCTCCTGTTGCTGCT-TCTGCTGTAAGCA- 44
Clone D195#2F (b) ------TTTAT-TAGCTGTTGCTCCTGTTGCTGCT-TCTGCTGTAATCA- 41
Clone D195#2C (c) ---TGCTTTAT-TAGCTGTTGCTCCTGTTGCTGCT-TCTGGCGTTGTTGC 45
Clone D255B (d)   TGCTGCTTTAT-TAGCTGTTGCTCCTGTCGCTGCT-TCTACTGTAAGCA- 47
Clone D256A (e)   ---TGCTTTAT-TAGCTGTTGCTCCAGTTGCTGCT-TCCGCTGTATCTA- 44
Clone D109E (f)   TGCTGCTTTATGTAGCTGTTGCTCCTGTCGCTGCTATCTGGCGTTG---- 46
Clone 44#2B (g)   ---TGCTTTAT-TAGCTGTTGCTCCAGTTGCTGCT-TCCGCTGTAT---- 41
Clone D42C (h)    ---TGCTTTAT-TAGCTGTTGCTCCTGTTGCTGCT-TCTGCTGTAAGCA- 44
                     ***** ************** ** ****** **    **

Clone D109D (a)   --CCGTTAACGCT------GCTAGTGTTACTTCCGCTACTCAATTAGGTA 86
Clone D195#2F (b) --CTGTTAACGCT------GCTGCCGTTACTACTGCTACTCAACTTGGTG 83
Clone D195#2C (c) TCCAGCTTCAGTTGTTTCAGCTGCTACTACTGCAGATGTTAAGGTACCAG 95
Clone D255B (d)   --CTGTTAATGCTGCTTCAAGTAGTGCTATTACTAGCGTTGGTTTAGGTA 95
Clone D256A (e)   --CTGTTTCAGCT------GATCCTGTTCAAACTGCTACTCAACTTGGTA 86
Clone D109E (f)   --TTGTCCAGCT------GCAACTNGTATCAN-GCTGATACTGTAAATG 87
Clone 44#2B (g)   --CAGTTAACGC------AGATGCAAATACTACTGTAAGTATCGGTAATT 83
Clone D42C (h)    --CTGTTAATGC------AGACGCTGTTACTAGTGTTGATCACTTAGGTA 86
                    * *         *                 *

Clone D109D (a)   A-GGTACCTACTTTAGCTAATGGTGATGCTGTAAATGT----TAAGCCAA 131
Clone D195#2F (b) G-TGTAAAATTACCAACTTCAAATGCTGCTGTAAATGT----TAAGCCAA 128
Clone D195#2C (c) GTGTTATTACTTTACACCGTGGCACCAACAGCCAATTTACTCTTAGCTTG 145
Clone D255B (d)   G-GGTTGAATTA---AGCAATGGTGACGTTGTTACTAT----TAAGCCTA 137
Clone D256A (e)   C-TGTACCTGCTTTATCAAACGGTGACACTGTTAACGT----TAAGCCAA 131
Clone D109E (f)   CTAGTAACAGTACTTGCAGACTACTCACACATTAACTTGGG-TGGTTCAG 136
Clone 44#2B (g)   C-ATTAACTCCATTACCAGACAACTCAACTGTAAAGCT----TTCATC-A 127
Clone D42C (h)    G-TGTTACTTTACCTAACAACGGTGCTGTTGTTAACGT----TAAGCCAA 131
                    *                       * *   *

Clone D109D (a)   ATGTTTCATTAAACACA---GTACATGGTTCA-ATTAAGGCAGCTATTTC 177
Clone D195#2F (b) ACGTTTCATTAAATACTGCTGTAGGTAACAGC-GTTAAGGCTTCAATTTC 177
Clone D195#2C (c) AATTCAACTCAAGAGGCTAGTTTATTACAACAACTTAAAGCTCCAGCTGG 195
Clone D255B (d)   ACATTAGCTTGAACAGTTCTGTAGGTAACGTT-GTTGCTGCTAACATTTC 186
Clone D256A (e)   ATGTTTCATTAAACACTTCAGCTTATGAAGGT-GTTAAGGCAAACATCTC 180
Clone D109E (f)   ACGTTA--CTAAGTATG-----TTGCAAGAC--GTTAACCCATCTATTTA 177
Clone 44#2B (g)   TCATTA--TCAGGTGTT---GTAAGCATGAAT-GGTGATGTAGCATACCC 171
Clone D42C (h)    ACATCAGCTTAAATACT---AAGGCTGACTCA-GTAGATGCTGCTATCTC 177
                    *                       *

Clone D109D (a)   TGTATCATTTGACGCTACTTTTAACGGTACTACTGCTACCTCAAACTTTA 227
Clone D195#2F (b) AGTATCATTTACTGCTACTATTGACGGTACTACTGCTACTGCTAACTTAG 227
Clone D195#2C (c) CGCAAAGATCAGCAATGTAGTTGTTAAGCAAGTTACTGCTACTAACCCAA 245
Clone D255B (d)   AGTATCATTCTCAGCTACTGTTAACGGCACTACCGCTACTTCAAACTTTA 236
Clone D256A (e)   AGTATCATTCTCAGCTGCTGTTAATGGTACTACCGCTATTTCAAACTTTA 230
Clone D109E (f)   CTTTAAACGCTGCA----TCTTCGTAAGAACAACGATAACACTAATGCAA 223
Clone 44#2B (g)   --TGACACTTTCCA----TGTTGGTGGTTCTATTTCTGCT---AACTTAG 212
Clone D42C (h)    AGTTTCATTCTCAGCAACTGTTAATGGTACTACTGCTAACGCAAACTTTA 227
                                    **          *     **

Clone D109D (a)   AGC----------CTGGATACTCA------AACATCCAACTTTTCCACGGT 262
Clone D195#2F (b) ATCCTAAGTCTACTGAAGTAAGTCTCTACAAGGGTACTGTTTCAGATGCA 277
Clone D195#2C (c) ATGGTGAATTTAACCAATACTTTACTTTTGATGTCGTAAATGAAAAGGGT 295
Clone D255B (d)   CACCAAACGCTTCAGAAGTTTCACTTTGGAAGACCGAAAACAATACCACT 286
Clone D256A (e)   AGCCAGGTGTTTCAGAAATTTCACTTTGGAAAGTTGAAAAGGACAAGGTT 280
Clone D109E (f)   ATGCN-----T--CAAGCTGTTGCTGCAGGTAGCTTGAACTGGTAGTGTT 266
Clone 44#2B (g)   CAGGTAGC------AACTTCTCA------GCTATTTTACCAGCTGATGCT 250
Clone D42C (h)    GTGTTAACCACACTAACCCTTCA------AACATTCAACTTTTCAAGGGT 271
```

D.

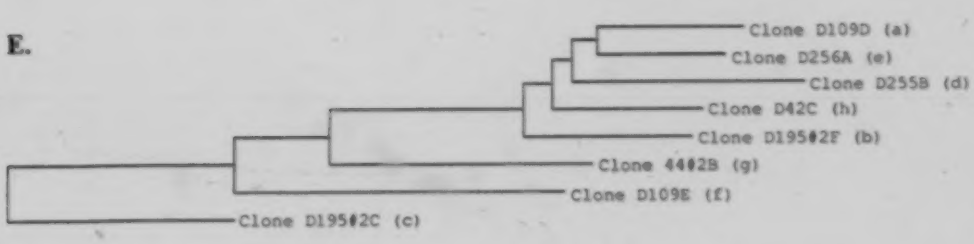
```
Clone D109D (a)    AGCAAGG--AAATTACT-AATTTACAAGATGT------------TCATTA 297
Clone D195#2F (b)  AATAAAG-----TTACC-GACTTACAAGACGT------------AACTTC 309
Clone D195#2C (c)  GAAGTTC-----TTAAG-AGTGCTACTGGTATGACTA---A---TGCTAA 333
Clone D255B (d)    ACTGAAATTAAGCCAAATAATTTGAATGATGTAGTTTCTCACTATGCTGG 336
Clone D256A (e)    ACTCACG-----T-AACTGATTTACAAAAAGT------------AACTTC 312
Clone D109E (f)    ACTGCTA--ACGTTGGTGGACGTAAGCA----------------TGCTAC 298
Clone 44#2B (g)    AACATGG-----TTGCT-ACTGCAAACAATACTGC----------CGATCA 285
Clone D42C (h)     AGCGAAG--AAATTAAA-GACTTAAACAAAGT------------TACTGA 306
                                                                *

Clone D109D (a)    CCTTACTGCAGGTTCA---------ACTTACCATGTTGTAATGAGCC-AT 337
Clone D195#2F (b)  AGCTGATGCTGGTACA---------GAATATAAAGTTGGTATGAACA-AC 349
Clone D195#2C (c)  GTTTGCTAGTCAACAAGGTACTCCATACTTCTACAATATTGCAGATA-AC 382
Clone D255B (d)    TGCTGATGGCAGAAAGCATCACGCTACTTTCCAAGTTAAGATGACCA-AG 385
Clone D256A (e)    ATCAAACGCTGGTGCT---------ACTTACCAAGTTAAAATGACTA-AT 352
Clone D109E (f)    TGCTAANCTTAGTTAA----------CGGTAGATCACGGTG--CAGCT 334
Clone 44#2B (g)    AGGTAAACCAGGTAAA---------TACACTGTAGCATACCCAGCAGAT 325
Clone D42C (h)     AGCAGATGCTGGTCAA---------ACTTACAAGGTTTCAATGACTA-AT 346

Clone D109D (a)    GTTGGTTTAAACTTTGGTTCACAAAACGCAAACAAGGAAATCACTTTAAC 387
Clone D195#2F (b)  GTTTCTTTAAACTTTGGTTCACAAAATGCTAACAAGAAAGTTACTTTGAC 399
Clone D195#2C (c)  ACTGTTATCAACAATGGTAGCACTGTAACTATCAATGATATTGCAAACGG 432
Clone D255B (d)    GTAGGTTTGAACTTTGGTACTCAAAACGCTAATAAGAAGATGACTTTAGC 435
Clone D256A (e)    GTTGGTTTGAACTTTGGCTCACAAAATGCTAACAAGAAGATTACTTTAAC 402
Clone D109E (f)    AACGTTAAGGTTACTGCTGTACAAGGCGGTA-CTGTAATNTTATGATGG- 382
Clone 44#2B (g)    ACTTCTATTAACTTTGGTACTTCAAATGCTAACAAGGAAGTTACTATCAC 375
Clone D42C (h)     GTTGGTTTGAACTTTGGTTCACAAAACGCTAACAAGAAAGTTACTTTGAG 396
                        *       **              *     * *

Clone D109D (a)    TATGCCTGAAGGCGAT-----TTCTTCCAACTTGCATCAAACAATACTGT 432
Clone D195#2F (b)  TTTCCCAGATAACGGA--------TCACACTTTGGCTACAATGGTAAAGA 441
Clone D195#2C (c)  CTTCACTCCATCTTCACTTTTGAAGGAAATTAACGATAAGTATGCATGGA 482
Clone D255B (d)    TGCACCTACTGCTGAT--GGTTACCTTAAGCATGG-TAGCAAGAGCGGTC 482
Clone D256A (e)    TTTCCCAGAAGGTGAT-----GGCTTTAAGCTTGCTTCAAACAACTCATT 447
Clone D109E (f)    TACTGATGCTGATCAC------GTCGTATCAACACTTCAAGCGCTGTAGT 426
Clone 44#2B (g)    TGTTCCAGCAGGTATG------GTAGCTACTGTNTCTACAGATAACTACG 419
Clone D42C (h)     CTTTGGTTCTGACAACTGGGCTGCACATAATGCGACTGATGCAATGAAGC 446
```

E.



F.

	Clone D109D (a)	Clone D1952#F (b)	Clone D195#2C (c)	Clone D255B (d)	Clone D256A (e)	Clone D109E (f)	Clone D44#2B (g)	Clone D42C (h)
Clone D109D (a)		66	8	64	74	9	13	65
Clone D1952#F (b)			13	51	68	51	55	80
Clone D195#2C (c)				12	14	18	8	16
Clone D255B (d)					66	12	14	66
Clone D256A (e)						57	67	69
Clone D109E (f)							16	17
Clone D44#2B (g)								13
Clone D42C (h)								

A.

```
Clone D109D (a)   ALLAVAPVAAS-AVSTVNAASVTSATQLGKVPTLANGDAVNVKPNVSLNT 49
Clone D256A (e)   ALLAVAPVAAS-AVSTVSADPVQTATQLGTVPALSNGDTVNVKPNVSLNT 49
Clone D195#2F (b) -LLAVAPVAAS-AVITVNAAAVTTATQLGGVKLPTSNAAVNVKPNVSLNT 48
Clone D195#2C (c) ALLAVAPVAASGVVAPASVVSAATTADVKVPGVITLHRGTNSQFTLSLNS 50
                   ********** .* .... .. :::::      :    .* : .:***:

Clone D109D (a)   -VRGSIKAAISVSFDATFN------------------------------- 67
Clone D256A (e)   SAYEGVKANISVSFSAAVN------------------------------- 68
Clone D195#2F (b) AVGNSVKASISVSFTATID------------------------------- 67
Clone D195#2C (c) TQEASLLQQLKAPAGAKISNVVVKQVTATNPNGEFNQYFTFDVVNEKGEV 100
                      .:   :...  * ..                  .

Clone D109D (a)   --GTTATSNFKPGYSNIQLFHGSKE----ITNLQDVHYLTAG-------- 103
Clone D256A (e)   --GTTAISNFKPGVSEISLWKVEKDKVTHVTDLQKVTSSNAG-------- 108
Clone D195#2F (b) --GTTATANLDPKSTEVSLYKGTVSDANKVTDLQDVTSADAG-------- 107
Clone D195#2C (c) LKSATGMTNAKFASQQGTPYFYNIADNTVINNGSTVTINDIANGFTPSSL 150
                    .:*. :* .    :   :         :.: . *     .

Clone D109D (a)   ---------------STYHVVMSHVGLNFGSQNANKEITLT-------MP 131
Clone D256A (e)   ---------------ATYQVKMTNVGLNFGSQNANKKITLT-------FP 136
Clone D195#2F (b) ---------------TEYKVGMNNVSLNFGSQNANKKVTLT-------FP 135
Clone D195#2C (c) LKEINDKYAWKTSDASGSKAEMTTTASDVESQLVAQGLKRASNGDFDYPA 200
                                : :. *. .. :. ** . : :. :        .

Clone D109D (a)   EGDFFQLASNNTVTNSRTIKLDQNGTVTLNQVAINVTAKDFAN------- 174
Clone D256A (e)   EGDGFKLASNNSFTNSRTIQLDKNGTVTLNEVVLHVTAKDFAN------- 179
Clone D195#2F (b) D-NGSHFGYNGKDGRSQEVQLNKDGVVTLSYVIFPVTAKNFAN------- 177
Clone D195#2C (c) NGFNLKLSAKSENGNTASITVRINAVVNYNAPAFVVDNTVYFNNNVTVNG 250
                  :    ::. :.   .:  : :  :..*. .   : *  . : *

Clone D109D (a)   -------------PAVVAWYDRETNVNVTSGNITL----DAGKMNVAQFV 207
Clone D256A (e)   -------------PAVVNWLNATTNAVVNSGNVELYAGSDAGKMNVNQVI 216
Clone D195#2F (b) -------------PEIVSWFNVTTGAPVTSASIQLYAGSNAGKMNVAQVI 214
Clone D195#2C (c) TTSGDLVIRDTKDTKVIVNDKLNEKAVTDAVTVYVQSAYGNSYDKTAKEL 300
                            . ::   .    . . : .: :    . .  :. : :

Clone D109D (a)   AAAENKYVARNNAGDKHNS-------------QSSTISYSNNIKEALKAM 244
Clone D256A (e)   AAVRAKYYATN-FGTLTNK-------------ESSDIKYTDNLVEALKAA 252
Clone D195#2F (b) SAVSPLENKGNGYVAMQYGN----------KLAFDTTANINNLKSALKAM 254
Clone D195#2C (c) SEHQQKINPSNIKIDSSAVNGKVAGMYPVTITATNPAGFTSKLVVYVIVM 350
                  :        *                      .     .::   : .

Clone D109D (a)   NIDIDANGWFVAPK-------------------------------SFTFE 263
Clone D256A (e)   GVEVKDN-WFVAPK-------------------------------SFTFN 270
Clone D195#2F (b) NIDVDANGWFVAPK-------------------------------SFTFN 273
Clone D195#2C (c) GPTEKVPAQYVHVDNAKVYNINGNVVTEDSSIKPLAKGAAVYAFDSVTVN 400
                  .   .    :*  .                                *.*.:

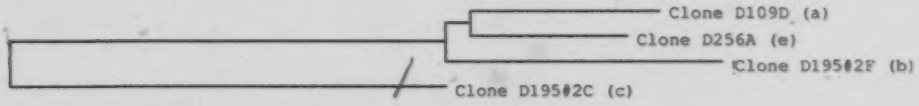
Clone D109D (a)   MTASANNNDASAKLPITVTVPNGKDVTLATVPSQSKTIMHNAYFYDKDAK 313
Clone D256A (e)   MTATANNNDASKTLAVTVNVPNGKEVTPATVPSQPKTIMHNAYFYDKDAK 320
Clone D195#2F (b) LTATSPKNDATATLAVTVNVPNGVNPTPATVPSQSKTIMHNAYFYDKDAK 323
Clone D195#2C (c) GVKYTEINSKGSNKWVKASDLTATKPAPAVVASQSKTIMHNAYFYDKDAK 450
                   .  :  *.   .  :... .. . : *.*.**.***************
```

Fig. 3.9. Comparison of 4 S-proteins encoded by the variable regions of *L. gallinarum* S-protein genes. **A**. ClustalW alignment. "*" indicates identical nucleotide in all sequences, ":" indicates very similar but different amino acids, "." indicates similar but different amino acids. **B**. Phylogenetic tree based on ClustalW protein sequence comparisons. **C**. Percent identity based on protein sequence comparisons.

A.

```
Clone D109D (a)    RVGTDKVTRYNTVTVAMNTTKLANGISYYEVIENGKATGKYINADNIDGTKRT 366
Clone D256A (e)    RVGTDKVTRYNTVNVSMNKTKFSNGIEYYEVIEGGKATGKFINADNIDGTKRT 373
Clone D195#2F (b) RVGTDKVTRYNTVTVAMNTTKFSNGIEYYEVIENGKATGKYINADNIDGTKRT 376
Clone D195#2C (c) RVGTDKVTRYNTVNVSMNKTKFSNGIEYYEVIEGGKATGKFINADNIDGTKRT 503
                  ************.*:**.**::***.******.******:************
```

B.



C.

	Clone D109D (a)	Clone D1952#F (b)	Clone D195#2C (c)	Clone D256A (e)
Clone D109D (a)		61	31	70
Clone D1952#F (b)			27	63
Clone D195#2C (c)				32
Clone D256A (e)				

A.

L. acidophilus ATCC 4356 SlpA
L. acidophilus ATCC 4356 SlpB
L. crispatus JCM 5810 CbsA
L. crispatus JCM 5810 CbsB
L. crispatus LMG 12003 SlpnA
L. crispatus LMG 12003 SlpnB
L. crispatus M247 S-layer
L. crispatus MH315 LbsA
L. crispatus MH315 LbsB
Clone D109D (a)
Clone D195#2F (b)
Clone D195#2C (c)
Clone D256A (e)

Fig. 3.10. Comparison of the variable regions of *Lactobacillus acidophilus* group S-proteins. **A**. ClustalW alignment. Boxed amino acids indicate signal sequence. Red boxes indicates potential signal sequence of *L. gallinarum* proteins based on homology with *L. acidophilus* and *L. crispatus* S-protein signal sequences. Blue boxes indicate areas of identity between a, b and e from *L. gallinarum* and other S-proteins. Green boxes indicate sequences unique to S-proteins a and b. Yellow boxes indicate surface exposed amino acids in SlpA [39]. Orange boxes represent regions important for S-layer formation for SlpA [39]. "*" indicates identical amino acid in all sequences. The GenBank accession numbers are as follows: ATCC 4356 SlpA, CAA61560; ATCC 4356 SlpB, CAA61561; JCM 5810 CbsA, AAB58734; JCM5810 CbsB, AAC28100; LGM 12003 SlpnA, AAF68971; LGM 12003 SlpnB, AAF68972; M247 S-layer, CAA07708; MH315 LbsA, BAC76686; MH315 LbsB, BAC76687. **B**. Phylogenetic tree based on ClustalW protein sequence comparisons. **C**. Percent identity of S-proteins based on protein sequence comparisons.

A.

```
L. acidophilus ATCC 4356 SlpA -------VK----PAELAAGVAYTVTVND-VSFNFGSENAGKTVTLGSAN 148
L. acidophilus ATCC 4356 SlpB -------EQ----INGLEPGSAVTVTLRDGVSFNFGSTNANKTITLAFPK 148
L. crispatus JCM 5810 CbsA    -L-----QQ----VTSSNAGATYQVKMTQ-VGLNFGSQNANKKVTLTFPE 158
L. crispatus JCM 5810 CbsB    -------DE----LANVNAGDTVTVSVAN-VGFNFGSENKGKKVTFKSSN 144
L. crispatus LMG 12003 SlpnA  -------DE----LSNVNAGSSNTITIKN-VGFNFGPENKGKKITLVSSN 157
L. crispatus LMG 12003 SlpnB  -L-----QK----VTSSNAGATYQVKMTN-VGLNFGSQNANKKITLTFPE 149
L. crispatus M247 S-layer     ----------------LTNGSYTVTVSG-VSFNFGTANANKTITLGSKN 157
L. crispatus MH315 LbsA       -ETKKVENN----LNNVVAGQSYTLTLTN-VGFSFGSAMKNKTVTVKLAN 169
L. crispatus MH315 LbsB       -L-----QQ----VTSSNAGATYQVKMTQ-VGLNFGSQNANKKVTLTFPE 152
Clone D109D (a)               -L-----QD----VHYLTAGSTYHVVMSH-VGLNFGSQNANKEITLTMPE 132
Clone D195#2F (b)             -L-----QD----VTSADAGTEYKVGMNN-VSLNFGSQNANKKVTLTFPD 136
Clone D256A (e)               SLLKEINDKYAWKTSDASGSKAEMTTTASDVESQLVAQGLKRASNGDFDY 198
                              -L-----QK----VTSSNAGATYQVKMTN-VGLNFGSQNANKKITLTFPE 137

L. acidophilus ATCC 4356 SlpA --SNVKFTGTNSDNQT-ETNVS---TLKVKLDQNG-VASLTNVSIANVYA 191
L. acidophilus ATCC 4356 SlpB NVSAAGLADANKVSATSETSVDAGKTIQVKTDKNG-VVSFGSAQVLNVKV 197
L. crispatus JCM 5810 CbsA    -GDMFK-TADTSLAQSHE----------VQLDKNGTTT-LPEV-VMNVTA 194
L. crispatus JCM 5810 CbsB    --SNVTFASSNSNAQVS----ADGKTVTATLDQNGTVSGLT-V-VERLVA 186
L. crispatus LMG 12003 SlpnA  --SKVTFGSDNA----------KTVTVSLDQNGTAKDLT-VNISDVTA 192
L. crispatus LMG 12003 SlpnB  -GDGFKLASNNSFTNSRT----------IQLDKNGTVT-LNEV-VLHATA 186
L. crispatus M247 S-layer     --SNVKFAGADGKF---------ADTVKVELGQNGTLTTPISVQVSNVNA 196
L. crispatus MH315 LbsA       --GELSGKNVTKNT---------DGSYKLTLDQYGNATELTYTQ--SLKA 206
L. crispatus MH315 LbsB       -GDMFK-TADTSLAQSHE----------VKLDQNGTIT-LPEV-VMNVTA 188
Clone D109D (a)               -GDFFQLASNNTVTNSRT----------IKLDQNGTVT-LNQV-AINVTA 169
Clone D195#2F (b)             NGSHFGYNGKDG--RSQE----------VQLNKDGVVT-LSYV-IFPVTA 172
Clone D195#2C (c)             PANGFNLKLSAKSENGNTASITVRINAVVNYNAPAFVVDNTVYFNNNVTV 248
Clone D256A (e)               -GDGFKLASNNSFTNSRT----------IQLDKNGTVT-LNEV-VLHVTA 174

L. acidophilus ATCC 4356 SlpA INTTDNSNVNFYDVTSGATVTN--------GAVSVNADNQ-GQ---VNVA 229
L. acidophilus ATCC 4356 SlpB VETSDVRAVSFYDIQTGKTVEN--------GTLSIVAGSN-AR---ANVQ 235
L. crispatus JCM 5810 CbsA    KNFANPTVVTWLNGTTSAPVTA--------GNITLYAGSDAGK---MNVA 233
L. crispatus JCM 5810 CbsB    YDATNTNDVVFYNIATGQPVNS--------GDAMVLADSN-KQ---LNVA 224
L. crispatus LMG 12003  SlpnA FNATNTNGVVFYNVTTGTQAHA--------GNAMVLANTQ-GQ---LNTA 230
L. crispatus LMG 12003 SlpnB  KDFANPAVVNWYNTATNSVVST--------GNIELFAGSDAGK---MNVA 225
L. crispatus M247 S-layer     LDLSNANGVNFYNASNGSQVTK--------GSVNVTAGLI-GR---LNVS 234
L. crispatus MH315 LbsA       YNQGNTNSVFFINQNSGTTETK--------GLYLTLANGN-GE---LNVA 244
L. crispatus MH315 LbsB       KDFANPAVVNWYNTATNAVVST--------GNIELFAGSDAGK---MNVA 227
Clone D109D (a)               KDFANPAVVAWYDRETNVNVTS--------GNITL----DAGK---MNVA 204
Clone D195#2F (b)             KNFANPEIVSWFNVTTGAPVTS--------ASIQLYAGSNAGK---MNVA 211
Clone D195#2C (c)             NGTTSGDLVIRDTKDTKVIVNDKLNEKAVTDAVTVYVQSAYGNSYDKTAK 298
Clone D256A (e)               KDFANPAVVNWLNATTNAVVNS--------GNVELYAGSDAGK---MNVN 213

L. acidophilus ATCC 4356 SlpA NVVAAINSKYFAAQYADKKLNT-----------R---TANTEDAI----- 260
L. acidophilus ATCC 4356 SlpB EIVNAFNAKYQASQLNNANSNA-----------NVRLTDNNAQAV----- 269
L. crispatus JCM 5810 CbsA    QVVAEAR------KNYVAM---GAKVA------DP------TNNI----- 257
L. crispatus JCM 5810 CbsB    AILPAVKSNFTATQRVTVAQGNGNGTYSQD-QINTVKINTTTPEI----- 268
L. crispatus LMG 12003 SlpnA  ALLPAIESNYVAVQRVDSDSANGNGTYNFA-DFKHVNNIEFATAI----- 274
L. crispatus LMG 12003 SlpnB  QVTSAAL------KKYHASNY-GTAAN------QESSTISYSNNL----- 257
L. crispatus M247 S-layer     TVASEILKNCAAYQ-----VSNGKPVSQLP-DQKAVV-----ADV----- 268
L. crispatus MH315 LbsA       DVLANIEKQYTAVQ-----YNDSKFMSSTE-KDSPVTITTNKDAV----- 283
L. crispatus MH315 LbsB       QVVSATE------KKYHASNY-GTKAN------QESSTISYTNNL----- 259
Clone D109D (a)               QFVAAAE------NKYVARNNAGDKHN------SQSSTISYSNNI----- 237
Clone D195#2F (b)             QVISAVSPLENKGNGYVAMQYGNKLAF------DTTANI---NNL----- 247
Clone D195#2C (c)             ELSEHQQKINPSNIKIDSSAVNGKVAGMYPVTITATNPAGFTSKLVVYVI 348
Clone D256A (e)               QVIAAVR------AKYYATNF-GTLTN------KESSDIKYTDNL----- 245
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A.

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L. acidophilus ATCC 4356 SlpA ----KAALKDQKIDVNSVGYFK-----------------APHTFTVNVKA 289
L. acidophilus ATCC 4356 SlpB ----ATMLRAQNIDVDAQGYFT-----------------APASLSLTFHA 298
L. crispatus JCM 5810 CbsA    ----KEALKAMNIDVDARGWFV-----------------APKSFTFNLTA 286
L. crispatus JCM 5810 CbsB    ----KDQLEKAGIKIDANGNFT-----------------APHSFKVTVKA 297
L. crispatus LMG 12003 SlpnA  ----KDQLKAQNIDVGPQGFFK-----------------APHTFTVKVKA 303
L. crispatus LMG 12003 SlpnB  ----VEALKAAGVEVK-DNWFV-----------------APKSFTFNMTA 285
L. crispatus M247 S-layer     ----NAALKAANIPVDNAGWFT-----------------APISLSVNVKA 297
L. crispatus MH315 LbsA       ----IAELKKQNIPVNAAGNFT-----------------APDTFTVTLNA 312
L. crispatus MH315 LbsB       ----KDALKAMNVDVDAQGWFV-----------------APKSFTFNMTA 288
Clone D109D (a)               ----KEALKAMNIDIDANGWFV-----------------APKSFTFEMTA 266
Clone D195#2F (b)             ----KSALKAMNIDVDANGWFV-----------------APKSFTFNLTA 276
Clone D195#2C (c)             VMGPTEKVPAQYVHVDNAKVYNINGNVVTEDSSIKPLAKGAAVYAFDSVT 398
Clone D256A (e)               ----VEALKAAGVEVK-DNWFV-----------------APKSFTFNMTA 273

L. acidophilus ATCC 4356 SlpA TSNT-----NGKSATLPVVVTVPN---VAEPTVASVSKRIMHNAYYY-DK 330
L. acidophilus ATCC 4356 SlpB ESTQ-----NNETAQLPVTVSVTNGKEVTPSTVDSVSKSFMHNAYYY-DK 342
L. crispatus JCM 5810 CbsA    KSDV-----NDATATLPVTVNVPNGKDTT---VPSQSKTVMHNAYFY-DK 327
L. crispatus JCM 5810 CbsB    TSDV-----NGKSKELPVTFTVANVAE---PTVASQSKMIMHNAYYYKED 339
L. crispatus LMG 12003 SlpnA  TSSI-----NGKSEELPVTFTVANVAD---PVVPSQPKTIMHNAYYYKED 345
L. crispatus LMG 12003 SlpnB  TANN-----NDASKTLAVTVSVPNGKDMT---VPSQSKTIMHNAFYY-DK 326
L. crispatus M247 S-layer     SSSI-----NGVGCYFTCTVNVANGKD---MTVPSQSKTIMHNAYYY-DK 338
L. crispatus MH315 LbsA       KSSI-----NGKTGQLVVTVSVPNGKK---TTVASQEKTIMHNAYYY-DK 353
L. crispatus MH315 LbsB       KANN-----NDASSTLAVTVSVPNGKDMT---VPSQSKTVMHNAFFY-DK 329
Clone D109D (a)               SANN-----NDASAKLPITVTVPNGKDVTLATVPSQSKTIMHNAYFY-DK 310
Clone D195#2F (b)             TSPK-----NDATATLAVTVNVPNGVNPTPATVPSQSKTIMHNAYFY-DK 320
Clone D195#2C (c)             VNGVKYTEINSKGSNKWVKASDLTATKPAPAVVASQSKTIMHNAYFY-DK 447
Clone D256A (e)               TANN-----NDASKTLAVTVNVPNGKEVTPATVPSQPKTIMHNAYFY-DK 317
                                       *                       *  ****  *

L. acidophilus ATCC 4356 SlpA DAKRVGTDSVKRYNSVSVLPNTTTIN-GKTYYQVVENGKAVDKYINAANI 379
L. acidophilus ATCC 4356 SlpB DAKRVGTDSVKRYNSVSVLPNTTTIN-GKAYYQVVENGKAVDKYINAANI 391
L. crispatus JCM 5810 CbsA    NGKRVGSDKVTRYNSATVAMSTTTI-KGKAYYEVIENGKATGKFINAANI 376
L. crispatus JCM 5810 CbsB    GTTRANNDKAKRYESVTVAMSTKKIG-DKNFYEVIKDGKATSMYINADNI 388
L. crispatus LMG 12003 SlpnA  GTTRANNDKAKRYESVNVAMSTKKIG-NKDFYEVIKDGKATGMYINADNI 394
L. crispatus LMG 12003 SlpnB  NGKRVGSDKVTRYNSATVAMNTTTI-NGKAYYEVIESGKATGKFINAANI 375
L. crispatus M247 S-layer     DAKRVGTDKLTRYNSVTVAMNTTTIN-GKAYYEVIENGKATGKFINADNI 387
L. crispatus MH315 LbsA       DAKRVGTDKVTRYNKVTVATSTTKIG-DKTYYEVIENGKATGKYINADNI 402
L. crispatus MH315 LbsB       NGKRVGSDKVTRYNSATVAMNTTTI-NGKAYYEVIENGKATGKFINAANI 378
Clone D109D (a)               DAKRVGTDKVTRYNTVTVAMNTTKLANGISYYEVIENGKATGKYINADNI 360
Clone D195#2F (b)             DAKRVGTDKVTRYNTVTVAMNTTKFSNGIEYYEVIENGKATGKYINADNI 370
Clone D195#2C (c)             DAKRVGTDKVTRYNTVNVSMNKTKFSNGIEYYEVIEGGKATGKFINADNI 497
Clone D256A (e)               DAKRVGTDKVTRYNTVNVSMNKTKFSNGIEYYEVIEGGKATGKFINADNI 367
                                     **  **    *            * *   ***     ******

L. acidophilus ATCC 4356 SlpA DGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKI 429
L. acidophilus ATCC 4356 SlpB DGTKRTLKHNAYVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKI 441
L. crispatus JCM 5810 CbsA    DGTKRTLKHNAYVYKSSKKRANKVVLKKGTEVTTYGGAYTFKNGKQYYKI 426
L. crispatus JCM 5810 CbsB    DGTKRTLKHNAYVYKTSKKRANKVVLKKGEEVTTYGGTYTFKNGKQYYKI 438
L. crispatus LMG 12003 SlpnA  DGTKRTLKHNAYVYKTSKKRANKVVLKKGDTVVTYGGTYTFKNGKQYYKI 444
L. crispatus LMG 12003 SlpnB  DGTKRTLKHNAYVYKSSKKRANKVVLKKGDTVVTYGGTYTFKNGKQYYKI 425
L. crispatus M247 S-layer     DGTKRTLKHNAYVYKTSKKRANKVTLKKGTEVTTYGGTYTFKNGKQYYKI 437
L. crispatus MH315 LbsA       DGTKRTLKHNAYVYATSKKRANKFVLKKGEEVTTYGGTYTFKNGKQYYKI 452
L. crispatus MH315 LbsB       DGTKRTLKHNAYVYKSSKKRANKVVLKKGTEVVTYGGAYTFKNGKQYYKI 428
Clone D109D (a)               DGTKRT                                              366
Clone D195#2F (b)             DGTKRT                                              376
Clone D195#2C (c)             DGTKRT                                              503
Clone D256A (e)               DGTKRT                                              373
                              ******
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A.

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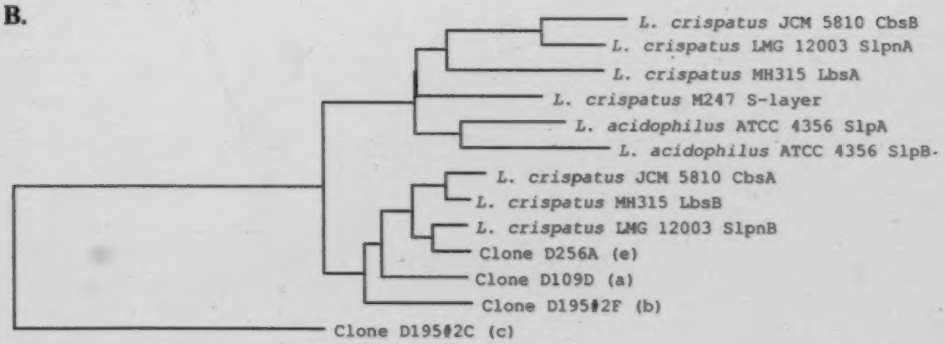
L. acidophilus ATCC 4356 SlpA GDNTDKTYVKVANFR 444
L. acidophilus ATCC 4356 SlpB GDNTDKTYVKVANFR 456
L. crispatus JCM 5810 CbsA    GNNTDKTYVKASNF- 440
L. crispatus JCM 5810 CbsB    GNDTKKTYVKASNF- 452
L. crispatus LMG 12003 SlpnA  YNNTEKTYVKASNF- 458
L. crispatus LMG 12003 SlpnB  YNNTEKTYVKASNF- 439
L. crispatus M247 S-layer     GNNTDKTYVKASNF- 451
L. crispatus MH315 LbsA       GNDTKKTYVKASNF- 466
L. crispatus MH315 LbsB       GNNTDKTYVKASNF- 442
Clone D109D (a)               --------------- 366
Clone D195#2F (b)             --------------- 376
Clone D195#2C (c)             --------------- 503
Clone D256A (e)               --------------- 373
```

B.


L. crispatus JCM 5810 CbsB
L. crispatus LMG 12003 SlpnA
L. crispatus MH315 LbsA
L. crispatus M247 S-layer
L. acidophilus ATCC 4356 SlpA
L. acidophilus ATCC 4356 SlpB
L. crispatus JCM 5810 CbsA
L. crispatus MH315 LbsB
L. crispatus LMG 12003 SlpnB
Clone D256A (e)
Clone D109D (a)
Clone D195#2F (b)
Clone D195#2C (c)

C.

	SlpA	SlpB	CbsA	CbsB	SlpnA	SlpnB	S-layer	LbsA	LbsB	a	b	c	e
SlpA		53	45	46	48	42	52	48	46	38	37	22	33
SlpB			41	36	36	41	44	41	43	30	36	22	34
CbsA				41	44	74	50	47	83	56	57	23	63
CbsB					70	41	45	47	43	33	30	21	31
SlpnA						42	48	48	44	31	32	23	30
SlpnB							48	45	84	64	54	22	82
S-layer								50	49	38	37	24	35
LbsA									46	37	37	23	30
LbsB										61	57	23	70
a											59	31	67
b												30	61
c													34
e													

BLAST-P results of translated proteins for S-protein types a, b, c and e are in Table 3.5 [1]. From published sequences, S-protein types a and e were most similar to SlpnB, while b was most similar to CbsA. S-protein type c was found to have no homology to S-proteins in the NCBI database, but did have low homology to a proteinase from *L. helveticus* over a short region (Table 3.5).

Although cloning of S-protein gene PCR products isolated two S-protein sequences for some strains (i.e. D109, D255 and D256 and perhaps D195#2 [refer to Discussion]), the presence of two S-protein genes was not confirmed for all strains. Total DNA in agarose plugs as used for PFGE (section 3.3.1.1) was digested with *Eco*RI. Fragments were separated by FIGE (Appendix Fig. A.3) and used in Southern hybridizations with probes to detect all S-protein genes and two specific S-protein genes (Table 3.2). The presence of two S-protein genes on unique sized DNA fragments was confirmed for representative isolates of PFGE groups as shown in Fig. 3.11. Hybridization with the universal probe clearly indicated that isolates D44#1, D42, D46, D73, D75#1, D108, D195#1, D195#2, 28-9, 31-5, D255, D256, D260#1, 15-6, 15-8, 15-9, 26-3, 28-1, 31-4 and 31-6 contained two homologous sequences to the 3' end of the S-protein genes. The positive control and isolates D41, D109 D148, 28-3, and 15-4 produced equivocal or negative results, although additional data as presented in the Appendix (Fig. A.6) indirectly indicated the presence of two unique S-protein genes for isolates D41, D109 and D148. Isolate 15-4 may have had too little DNA (Appendix Fig. A.3) to detect the hybridization of a radiolabelled probe (it has a faint PFGE pattern [Fig. 3.2]). Isolates 28-3 appeared to be poorly digested with *Eco*RI (Appendix Fig. A.3) and a smear was observed rather than two separate bands. The bands observed in Fig. 3.11

Table 3.5. BLAST-P results for translations of 4 completely sequenced S-protein variable regions cloned from *L. gallinarum* crop isolates.

Gene	Highest identity in BLAST-P (GenBank Accession no.)	Percent identity	e value
a	SlpnB (AAF68972)	67	$4e^{-96}$
	LbsB (AB11091)	65	$7e^{-95}$
	CbsA (AAB58734)	61	$3e^{-86}$
b	CbsA (AAB58734)	60	$3e^{-82}$
	LbsB (AB11091)	56	$7e^{-80}$
	SlpnB (AAF68972)	54	$5e^{-74}$
c	*L. helveticus* proteinase, aa 147-307 (BAB72065)	30	0.004
e	SlpnB (AAF68972)	81	e^{-127}
	LbsB (AB11091)	69	e^{-106}
	CbsA (AAB58734)	66	$4e^{-94}$

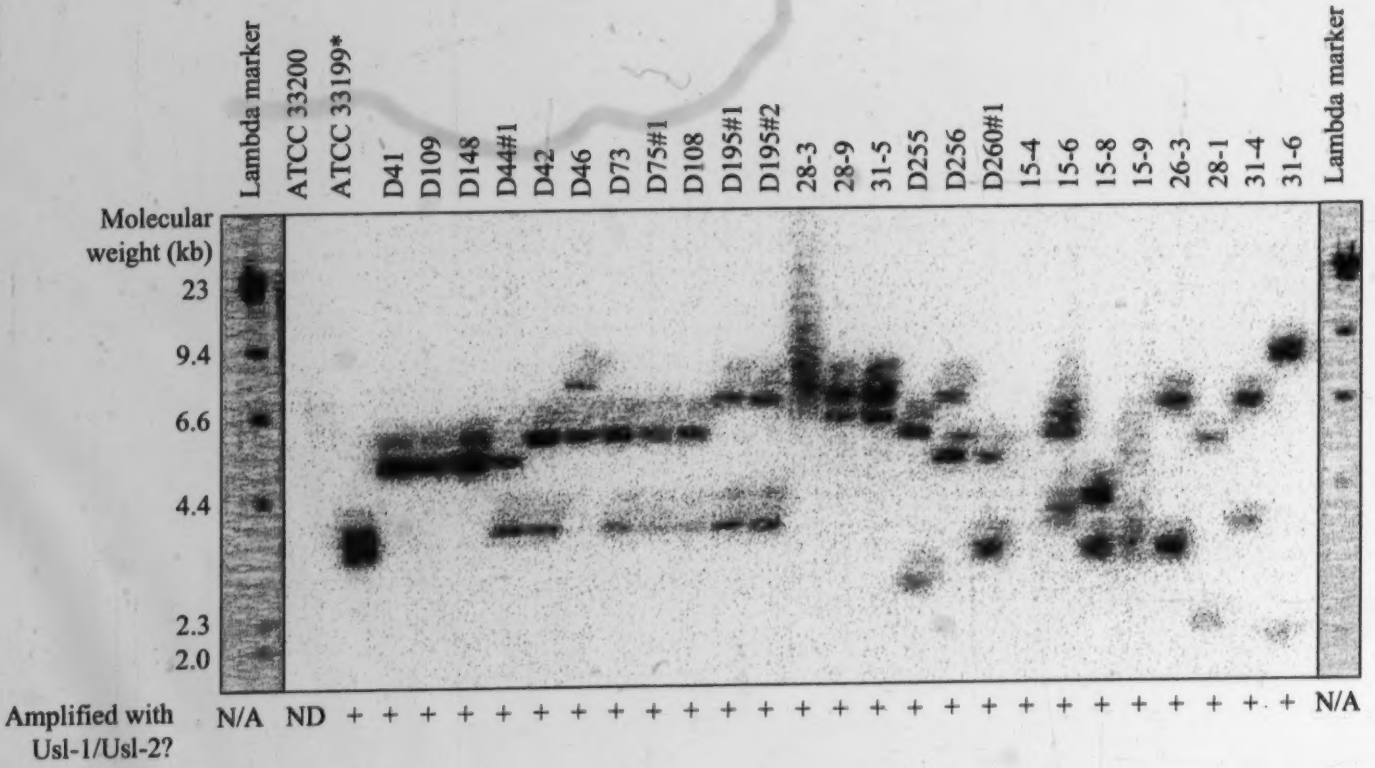


Fig. 3.11. Southern hybridization of chromosomal *Eco*RI digests of *L. gallinarum* isolates with universal S-protein probe (U). "*" indicates positive control. Text below figure indicates PCR results as presented in Table 3.6. "+" indicate PCR product, "-" indicates no PCR product. N/A - not applicable. ND - not done. Lambda markers derived from Fig. A.3 in the Appendix.

were confirmed to be S-protein genes by the hybridization of specific probes (discussed in section 3.3.4) in the same positions as the universal probe homologues. For example, the uppermost band of isolate D108 in Fig. 3.11 was at the same position as the band for the gene type a specific probe (Appendix Fig. A.4).

3.3.4. Prevalence of S-protein gene types among *L. gallinarum* isolates

Based on the sequences of the S-protein gene clones in section 3.3.3, gene specific oligonucleotides (Table 3.1) originally used for sequencing the genes with primer walking were also used to screen the *L. gallinarum* isolates (highlighted primers in Table 3.1). The results are summarized in Table 3.6. S-protein gene type a was found to be nearly universal; only PFGE groups 3 and 17 and the type strain lacked it. Interestingly, PFGE group 3 contained two unique gene types (b and c), which were not found in any other *L. gallinarum* isolates except for the presence of gene type b in PFGE group 17 and the *L. gallinarum* type strain. Gene types d and e were limited to the PFGE groups they were cloned from. Gene type f was found in PFGE group 1, 8 and 13. Gene type h was present in PFGE groups 2 and 4 and gave equivocal results for PFGE group 1.

The regions of S-protein gene types a and f, amplified by gene specific oligonucleotides (Table 3.2) were used as probes in a Southern hybridization. These results confirmed the PCR results listed in Table 3.6. The probe for gene type a detected homologues in isolates D41, D109, D148, D44#1, D42, D46, D73, D75#1, D108, 28-9, 31-5, D256, D260#1, 15-6, 15-8, 15-9, 26-3, 28-1, and 31-4 (Appendix Fig. A.4). Interestingly, isolate D255 lacked a hybrid for gene type a. The partial 5' sequences of S-protein genes of type a indicated slight differences (Appendix Fig. A.2), which may

Table 3.6. Summary of S-protein gene types found in *L. gallinarum* crop isolates

PFGE group	Gene type / Isolate	a	b	c	d	e	f	g	h
1	D41	+	-	-	-	-	+	-	+
	D109	+	-	-	-	-	+	-	+/-
	D148	+	-	-	-	-	+	-	*
2	D45	+	-	-	-	-	*	-	+
	D71	+	-	-	-	-	*	-	+
	D73	+	-	-	-	-	*	-	+/-
	D75#1	+	-	-	-	-	-	-	+
	D79	+	-	-	-	-	*	-	+
	D80#2	+	-	-	-	-	-	-	+
	D108	+	-	-	-	-	-	-	+
3a	D149#1	-	+	+	-	-	-	-	-
	D149#2	-	+	+	-	-	-	-	-
	D195#2	-	+	+	-	-	-	-	-
	D195#3	-	+	+	-	-	-	-	-
	D197#1	-	+	+	-	-	-	-	-
	D197#2	-	+	+	-	-	-	-	-
3b	D195#1	-	+	+	-	-	-	-	-
4a	D42	+	-	-	-	-	*	-	+
	D47	+	-	-	-	-	-	-	+
4b	D46	+	-	-	-	-	*	-	+
5	D44#1	+	-	-	-	-	-	+	-
	D44#2	+	-	-	-	-	-	+	-
6	D260#1	+	-	-	-	-	-	-	-
	D260#2	+	-	-	-	-	-	-	-
7	15-5	+	-	-	-	-	*	-	-
	15-8	+	-	-	-	-	*	-	-
8	28-1	+	-	-	-	-	+	-	-
	28-6	+	-	-	-	-	+	-	-
	28-7	+	-	-	-	-	+	-	-

Legend:

+ Large amount of PCR product (thick band)

* Product of correct size observed; absent after addition of 7% (v/v) DMSO to the PCR reaction or melting temperature of 69°C

+/- Small amount of PCR product (faint band) observed under highly stringent conditions (above)

□ Strain gene type was cloned from

Table 3.6. Continued

PFGE group	Gene type / Isolate	a	b	c	d	e	f	g	h
	28-3	+	-	-	-	-	*	-	-
	28-4	+	-	-	-	-	*	-	-
	28-9	+	-	-	-	-	*	*	-
9	31-2	+	-	-	-	-	-	-	-
	31-5	+	-	-	-	-	-	-	-
	31-7	+	-	-	-	-	-	-	-
	31-9	+	-	-	-	-	-	-	-
10	D255	+	-	-	+	-	-	-	+/-
11	D256	+	-	-	-	+	-	-	+/-
12	15-4	+	-	-	-	-	*	-	-
13	15-6	+	-	-	-	-	+	-	-
14	15-9	+	-	-	-	-	*	-	-
15	26-3	+	-	-	-	-	*	-	-
16	31-4	+	-	-	-	-	-	-	-
17	31-6	-	+	-	-	-	-	-	-
18	ATCC 33199	-	+	-	-	-	-	-	-

Legend:

+ Large amount of PCR product (thick band)

* Product of correct size observed; absent after addition of 7% (v/v) DMSO to the PCR reaction or melting temperature of 69°C

+/- Small amount of PCR product (faint band) observed under highly stringent conditions (above)

☐ Strain gene type was cloned from

explain the results. The gene type a probe was amplified from D109 template DNA, and the partial 5' sequence identity obtained from clone D109D to clone D255A is 93.7%. As the probe was designed to hybridize in the 3' end of the gene where complete sequence for clone D255A was not obtained, it seems possible that the sequence of this gene was significantly different (i.e. based on the 5' sequence heterogeneity) to not bind the probe at sufficient levels to observe a band. The probe for gene type f detected homologues in isolates D41, D109, D148, 15-6 and 28-1 (Appendix Fig. A.5), although the DNA concentration for isolates 28-1 appears to be a bit low, thereby explaining the fainter band (Appendix Fig. A.3).

The detection of gene types a and f in isolates D41, D109, and D148 was co-located to the same position as the universal probe (Appendix Fig. A.6). Therefore, this information indirectly indicates that these isolates do contain two unique S-protein genes despite the fact that only one band was observed with the universal probe.

3.4 Discussion

L. gallinarum is a member of the *L. acidophilus* group that was observed to be present in the chicken crop (Chapter 2). Genetic typing using PFGE (and to some extent, RAPD) indicated heterogeneity among *L. gallinarum* isolates, and a total of 17 different strains were detected among 44 isolates (Table 3.3, Figs. 3.2 & 3.3). PFGE was found to be consistent and reliable for typing the isolates, and while RAPD was a quicker method, it had some disadvantages as discussed below.

In seven strains, multiple colony types were observed (Table 3.3). In four strains (D44, D149, D197 and D260) the same PFGE pattern was observed for the colony variants, suggesting that the bacteria were from a pure culture and their growth *in vitro* may have induced expression of different surface components in a subpopulation of the culture. Differences in surface characteristics could be caused by extracellular polysaccharide (EPS) [36], expression of different S-proteins [21] or changes in expression of other surface proteins or cell wall components. In the case of D195, the *Sma*I-PFGE pattern of D195#1 showed a very slight difference in the migration of high molecular weight DNA fragments (noticeable but not obvious in Fig. 3.2). This could indicate that the variant is a mutant, although the *Apa*I digests did not cut the DNA for this group, thereby the observation could not be confirmed with a second digest. Altered running conditions for *Sma*I-PFGE or the choice of a different restriction enzyme could allow elucidation of the extent of mutation further. Isolate D46 may also be a variant of D42 and D47, as its pattern was similar but slightly different than theirs.

In two cases (D75 and D80), *L. gallinarum* isolates were co-purified from *L. crispatus* isolates obtained from the same culture. It is possible that the cultures were

mixed upon isolation, either due to close proximity when originally plated on LBS agar, or perhaps due to physical interactions between the bacteria. It has been shown that members of the *L. acidophilus* group aggregate when grown in broth culture [7,9,14,24,42], and it is possible that intra-species aggregation could occur in the GIT or upon isolation, resulting in some mixed colonies. It is interesting that the mixed cultures also contained the same strain of *L. gallinarum*. While this possibility has not been investigated further in this paper, it is intriguing to consider mixed species aggregates in the GIT environment and how that might affect persistence and colonisation.

Lactobacilli, including members of the *L. acidophilus* group are known to contain plasmids [25]. The potential functions of *Lactobacillus* plasmids is not well elucidated, although there is some evidence for correlation of antimicrobial resistance [43] and bacteriocin production [22,23] with the presence of plasmids. Plasmid profiles of *L. gallinarum* isolates (Fig. 3.3) generally agree with strain identifications as determined by PFGE (Fig. 3.2). An exception is seen with isolates 15-4, 15-5, 15-8 and 15-9 and PFGE group 9. Although 15-5 and 15-8 were typed as the same strain by PFGE a different plasmid profile characterised by fewer bands was observed in 15-5, while 15-4 (from another PFGE group) had a plasmid profile similar to 15-8. Isolate 15-9 also had a plasmid profile somewhat similar to that of 15-4. Isolates belonging to the same strain as determined by PFGE had different plasmid profiles when isolated from different chicken crops (PFGE group 9, Fig. 3.3). This observation is interesting as it may suggest that plasmid transfer can occur between different *L. gallinarum* strains in the crop. No data have been published regarding plasmids in *L. gallinarum* [25], and they could be interesting to investigate further.

Based on the data presented in Figs. 3.2, 3.3 & 3.4, different groupings of isolates were obtained. It is known that RAPD is very sensitive to PCR conditions, resulting in pattern variations between reactions [30], and indeed, other researchers have also found different groupings based on PFGE and RAPD [41]. With respect to plasmid profiles, the same strain could have the same profile, but different strains were likely to have the same profile if they were isolated from the same crop. Therefore, while PFGE was a more reliable typing method than RAPD, it was unable to detect differences in plasmid content. This is undoubtedly due to the small sizes of the plasmids observed in Fig. 3.4. While it is possible that larger (>20 kb) plasmids exist in our *L. gallinarum* strains, they would be undetectable in Fig. 3.4. Further, given most PFGE groups had highly similar patterns above 97 kb but many smaller bands below 97 kb (Fig. 3.2), it seems possible that plasmids of 20-90 kb could be present but would be unnoticeable.

SDS-PAGE profiles of *L. gallinarum* isolates revealed that isolates of the same strain produced similar sized S-proteins (Fig. 3.6). PFGE group 3 produced the largest S-proteins (estimated at 52.6 kDa), while PFGE group 2 produced the smallest S-proteins *in vitro* (estimated at 43.2 kDa). As discussed in Chapter 1 (section 1.3.2.2), *L. acidophilus* has been shown to undergo DNA recombination to express the otherwise silent S-protein gene (*slp*B) [3,4,6]. Boot et al. [5] showed that other members of the GAA carried the homologous recombination region originally identified in *L. acidophilus* ATCC 4356 (Chapter 1 Fig. 1.4). Indeed, the region upstream of *lbs*A from *L. crispatus* MH315 is very similar to that of *slp*A from *L. acidophilus* ATCC 4356 over almost the entire sequence, and appear to have the same promoter sequence (Chapter 1 Fig. 1.4). The partial sequences shown in Fig. 1.4 (Chapter 1) are also homologous to that of *slp*A.

Therefore, while the potential for DNA recombination and differential S-protein expression has not been demonstrated in other GAA isolates to date, it appears likely to occur. Our isolates were shown to have two S-protein genes as shown by PCR screening (Table 3.6), Southern hybridization (Fig. 3.11), and cloning (Table 3.4). Therefore, the identity of the S-proteins expressed *in vitro* as detected by SDS-PAGE in Fig. 3.6 cannot be stated with certainty, although it is possible to make predictions based on the sizes of the cloned S-protein genes when combining the SDS-PAGE and molecular data (below).

Cloning and sequencing of the S-protein genes resulted in four unique complete variable region sequences and four additional partial variable region sequences. Of the four complete sequences, three (a, b, and e) were somewhat similar, but the fourth sequence (c) was very dissimilar. BLAST-P analysis revealed low homology to a proteinase from *L. helveticus* (aside from high homology in the C-terminus, as expected) (Table 3.5). This predicted protein has a C-terminus that is almost identical to that of S-protein type e (Fig. 3.10A). The gene was therefore undoubtedly cloned due to its homology in the 5' and 3' ends where the primers anneal. It is difficult to determine the nature of this protein; its low homology to other S-proteins suggests that protein c may be something different altogether. No proteinase sequences have been published from *L. gallinarum*, thus it is difficult to determine if protein c could be a cell surface associated proteinase. The predicted size for protein type c is *ca.* 57 kDa and the observed major protein species is 52 kDa, which would suggest that the major band observed in Fig. 3.6 is different from that the predicted protein obtained from gene type c sequence. While *L. helveticus* proteinases are cleaved in the proprotein form to produce a smaller, mature protein [31] and an *L. gallinarum* proteinase of ca. 57 kDa could be cleaved to produce a

52 kDa protein, it seems unlikely that a proteinase would be expressed at the same level as S-proteins and thus a fainter band would be expected in SDS-PAGE. N-terminal sequencing or tandem mass spectrometry could reveal partial protein sequence and clarify if the expressed protein is different from the cloned gene. These data suggest that the Usl-1/Usl-2 primer pair may be non-specific for S-proteins and may amplify other genes in the genome where a C-terminal anchor is encoded. However, the partial sequences obtained appear to encode S-proteins due to their relatively high homology among each other (Fig. 3.8D & E). Gene type f has highest identity with gene type e (57%), as does gene type g (67%) (Fig. 3.8F). Gene type h has highest identity (80%) with gene type b. While complete sequences could not be obtained for these cloned S-protein variable regions, future work to complete the sequences could reveal unique motifs in the completed sequences.

Combining the data from Fig. 3.6 and the sequenced S-protein genes, it is possible to make some tentative assumptions about which of the cloned genes are expressed *in vitro*. For example, PFGE groups 2 and 4 produced S-proteins of similar sizes, and were found to carry the same gene (type h) by PCR screening. Therefore, it is possible that they both expressed the same protein (h) *in vitro*. The complete sequence for gene type h is not available at present, but if it encodes a protein of *ca.* 43 kDa, this would support the hypothesis. Two genes were cloned from isolate D109, the full sequence of gene type a is available but only the partial sequence of gene type f was obtained. The PCR-product observed from the clone of D109E was larger than that of the other S-proteins (data not shown) and may correlate to the large protein expressed by PFGE group 1. Indeed, PFGE group 8 also expresses an S-protein of the same size (Fig. 3.6D) and was found to carry

gene type f (Table 3.6). Isolate 15-6 was revealed to carry the gene type by Southern hybridization (Appendix Fig. A.5) and PCR screening (Table 3.6) but produced a different sized S-protein as seen in Fig. 3.6B. As discussed above, the gene type c may not correlate to the observed protein for PFGE group 3 (Fig. 3.6A), but it is clear that gene type b is too small (estimated as *ca.* 43 kDa) to be the expressed protein observed (52.6 kDa).

An alternative explanation for the varied S-protein sizes when detected by SDS-PAGE could be post secondary modifications such as glycosylation, addition of other functional groups and cleavage of peptide sequences from proproteins. S-proteins can be glycosylated [34], however it has been shown that *L. acidophilus* SlpA is non-glycosylated [3] and only non-GAA lactobacilli have been found to be glycosylated to date [29]. There is no evidence for proprotein cleavage with GAA S-proteins. The fact that the sizes of the proteins agree well with the sizes of the PCR products suggests that the proteins may vary in size based on primary structure alone. This could be investigated further by certain staining techniques of native cells or SDS-PAGE products [35] but it would be difficult to distinguish the carbohydrates attached to S-proteins from those of the EPS and the cell wall. Therefore, while it seems fairly unlikely that glycosylation of S-proteins occurs in the *L. gallinarum* isolates, if a particular S-protein is selected for future studies it might be advisable to have it tested in a fashion similar to that performed by Möschl et al. [29] to confirm the absence of glycosylation.

Comparison of S-protein sequences for *L. acidophilus*, *L. crispatus*, and *L. gallinarum* revealed several areas of homology highlighted in blue in Fig. 3.10A. These regions correspond well with the areas of SlpA that, when mutated, resulted in poor S-

layer formation ([39] orange boxes Fig. 3.10A). Areas of low or no homology were seen interspersed between the homologous regions, which also correlates well with the data generated by Smit et al. ([39] yellow boxes Fig. 3.10A). The valine-rich regions highlighted in blue in Fig. 1.7A (Chapter 1) fall with the conserved regions in Fig. 3.10A (data not shown). Comparison of complete S-protein variable region sequences from *L. gallinarum* isolates to published GAA S-protein sequences in GenBank reveals areas of conservation which are predicted to be responsible for mature S-protein folding and/or S-layer assembly. These regions are interspersed with variable sequences which are proposed to be surface exposed and therefore responsible for specific interactions with the environment, such as tissue adherence.

Screening of *L. gallinarum* isolates for the presence of the unique S-protein gene types by PCR revealed that gene type a was very well conserved (Table 3.6). This was confirmed by Southern analysis (Appendix Fig. A.4). Southern analysis and PCR screening also confirmed the prediction that *L. gallinarum* isolates carry two S-protein genes. The only data that contradicts this are the results for PCR screening of gene type h, where faint products were observed for isolates D109, D148, D255 and D256 and a strong product for D41, which were otherwise found to carry two S-protein genes (Table 3.6). The sequence for gene type f is incomplete and therefore cannot be compared to Fsl-7, although Rsl-7 does show homology to the 3' end of gene type f (data not shown), but not to gene types a, d or e. Therefore, the explanation for the faint products of similar size is unknown, although it is possible the primers detected homologous sequences elsewhere in the genome. Experiments could be conducted to investigate this phenomenon further. The Usl-1/Usl-2 and/or Fsl-7/Rsl-7 PCR products from D41 could be cloned and

sequenced. Hybridization with the probe for S-protein type h could also confirm the presence of absence of the gene in D41, D109, D148, D255 and D256. Further, primers specific to other regions in gene type h (when fully sequenced) could be designed and the isolates could be screened accordingly.

Cloning of Usl-1/Usl-2 PCR products amplified from *L. gallinarum* ATCC 33199 indicated that this strain carries gene type b (data not shown). This was confirmed with PCR screening (Table 3.6). This discovery is significant, as this particular S-protein type is therefore present in different strains from completely different flocks. Interestingly, PCR screening indicated that gene type b was mutually exclusive with gene type a; there were no isolates detected that carried both genes. The reason for this is unknown, but it is an intriguing observation. Analysis of the sizes of expressed S-proteins (above) suggests that PFGE groups 1, 8, and 3 are not expressing either S-protein a or b *in vitro*. Therefore, it is very interesting to hypothesize that these proteins are differentially expressed in the chicken GIT mediated by homologous recombination as initially discovered by Boot et al [6].

The data generated by this experiment has revealed that despite significant genetic diversity among *L. gallinarum* isolates, they carry at least one conserved S-protein gene. This finding is very important for future studies on persistence factors such as S-proteins, and indicates their presence may be necessary for GIT survival.

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Chapter 4: General Discussion and Conclusions

Our research has investigated the microecology of the chicken crop on a number of levels. Our pilot experiment (1st chicken experiment) was conducted to refine sampling techniques and obtain *Lactobacillus* isolates for future analysis. In our 2nd chicken experiment, we studied the chickens over the entire 42 days of the broiler production period, and investigated the chicken crop bacterial population with culture-dependent and culture-independent techniques. In particular, polymerase chain reaction (PCR) of 16S rDNA followed by denaturing gradient gel electrophoresis (PCR-DGGE) was used to discriminate among lactobacilli, and selective enumeration of crop homogenates allowed cultivation of lactobacilli (Chapter 2). A typing technique developed by Leluo Guan (Chapter 2 section 2.3.4) was used to speciate all isolates from the 1st experiment, and selected isolates from the 2nd experiment.

PCR-DGGE revealed that the most intense bands in crop profiles correlated to reference *Lactobacillus* strains, and pooled PCR-DGGE data suggest that *Lactobacillus crispatus* and/or *Lactobacillus gallinarum* (which could not be differentiated by PCR-DGGE) were present in the crop at all sampling times. This reflects individual crop profiles, which indicated that most chickens had *L. gallinarum* and/or *L. crispatus* (data not shown). We cultivated a large number of *L. gallinarum* isolates from crop homogenates plated at high dilutions, which suggests that *L. gallinarum* is present in the crop in high numbers. Therefore, *L. gallinarum* is a member of the crop microflora that is well represented and consistently detected through the production period. In chickens older than day 0, *Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Lactobacillus*

salivarius were also detected in PCR-DGGE patterns, and isolates from these species were obtained.

PCR-DGGE data of pooled crop profiles indicated that the composition of the *Lactobacillus* population stabilized after day 14, which was correlated to increased intensity of *L. salivarius* bands and decreased intensity of the bands for other bacteria (*L. reuteri*, *L. johnsonii* and *L. acidophilus*). This agreed with the PCR-DGGE data from individual birds; individual profiles had *L. salivarius* more frequently after day 14; fewer profiles from younger birds had *L. salivarius* (data not shown). Therefore, our data from young birds indicates that *L acidophilus* group members and *L. reuteri* are frequently present in significant numbers, and older birds are more likely to have *L. salivarius* co-exist in their crops along with the other *Lactobacillus* species detected. Indeed, Fuller [4] obtained a crop isolate which was likely *L. salivarius*, and Sarra [17] obtained a number of *L. salivarius* isolates from chicken crops. We cultivated *L. salivarius* isolates from young chickens (from day 5 onward in the 1st experiment) and *L. salivarius* was present in some individual PCR-DGGE profiles of birds 3 and 7 days of age (data not shown). This pattern of succession is interesting, and may reflect changes to the chicken crop tissue surfaces or to the crop environment as a whole. It is intriguing to speculate if *L. salivarius* could establish a population in older birds if the other species were not present. For example, could late colonizing species or strains adhere to the tissue directly, or to the cell surface structures of previously colonized bacteria? Could initial colonizers create an environment suitable for late colonizing species (i.e. production of sufficient essential metabolites, or reduction of species that could inhibit late colonizers)? In addition, could secondary metabolites produced by initial colonizers induce expression of

survival and/or persistence factors in late colonizers? The crop environment may also change due to developments in the physiology of the crop itself. Does sloughing of crop cells remain constant throughout the life of the bird, or would the rate increase or decrease in older chickens? Could the expression of cell surface (glyco)proteins or glycolipds on the crop epithelium change with age, resulting in different sites and mechanisms for adherence?

PFGE data suggest that different strains of *L. gallinarum* can co-exist within the same chicken crop, along with other species (as shown by PCR-DGGE profiles and the identification of the isolates). Interestingly, the bands for *L. reuteri* were observed for the PCR-DGGE profile of chicken 31 although no *L. reuteri* were cultivated. This can likely be explained by the fact that we did not select colonies randomly, but selected for colonies with unique morphologies. As observed with our *L. gallinarum* isolates, different strains of the same species can have different colony morphology, therefore selecting on the basis of colony type may have enriched for species that have variable surface characteristics.

Our data suggest that chickens of different ages can carry high populations of some strains, however we did not cultivate identical strains from the different flocks used in the two experiments. The chickens were obtained from the same supplier and were raised in the same research station. This observation is important, as it would suggest the bedding, drinking water and/or feed may be the source(s) and/or reservoirs of *L. gallinarum* strains. The feed was mixed separately for each experiment and the straw bedding was changed between experiments; thus these environments are implicated as a potential source for unique *L. gallinarum* populations seen among flocks. *L. gallinarum*

could be present in the chicken crop on day 0. Although chicks rely on their yolk sac for nutrition during the first few days of life [5], they do commence drinking, pecking and feeding after hatch [11], thereby inoculating their crops with lactobacilli. Some of the strains isolated were present in chickens of different ages, which would suggest that the strains were either transferred between chickens or that the chickens were consistently exposed to them from an environmental source such as the feed. The *L. gallinarum* shed from one chicken could likely persist in the feed, water or bedding and therefore inoculate other chickens. Indeed, Lu et al. [13] detected lactobacilli in used poultry litter. The possible environmental reservoirs of *L. gallinarum* strains have not been investigated further, but it would be interesting to track the spread of strains in the broiler production environment using culture-independent techniques [13].

Fuller [4,7-9] obtained a *Lactobacillus* isolate that was likely a strain of *L. salivarius*, which appeared to adhere to crop tissue via a carbohydrate moiety that was associated with a protein. Although experiments to determine the adherence of our *L. gallinarum* isolates *in vivo* have not yet been conducted, it is possible that these strains would adhere to crop tissue via their S-protein.

Regardless of the genetic heterogeneity among *L. gallinarum* strains cultivated, two S-protein gene types, a and b, were observed to be present among isolates from both experiments as shown by cloning and sequencing, PCR screening and Southern hybridization. Gene type a was well conserved, and was found in most *L. gallinarum* isolates. Gene type b was less frequently detected, but was also present in the *L. gallinarum* type strain, a crop isolate [6]. The observation of these genes among isolates obtained from different flocks is very important, and suggests these genes provide a

selective advantage in the crop. S-proteins form a protective layer on the bacterial surface [19] and expression of certain S-proteins may be necessary for survival in the GIT to exclude degradative enzymes and harmful chemicals. Alternatively, S-layers, which are highly hydrophobic on the environmental surface [20] may cause aggregation of the bacteria [12] and protect them from the GIT environment. S-layers may also mediate adherence [19].

As members of the *L. acidophilus* DNA homology group A (GAA) carry two S-protein genes, we cannot determine which gene would be expressed *in vivo* without conducting further experiments. First, RNA could be extracted from crop tissue homogenates of gnotobiotic chickens associated with an *L. gallinarum* isolate (for example, D109) and analysed using reverse transcriptase (RT)-PCR to determine the presence of S-protein gene type a mRNA. In addition, a crop tissue sample from the same chicken could be fixed and sectioned for microscopy to confirm the adherence of the isolate tested and then to investigate the ability of the protein to directly mediate adherence to the crop tissue. Adherence could be detected by non-specific staining of crop tissue sections, as adherent *L. gallinarum* and the crop tissue would be in close proximity. Microscopy (fluorescence, as in [18] or immunogold labelling in TEM) could be used to detect the presence of S-protein type a between the bacteria and the crop epithelium, in an experiment analogous to Brooker and Fuller's early research [4]. This would require raising antibodies to the natively folded S-protein as well as antibodies for chicken crop tissue.

Several other techniques could be employed to determine the role of the expressed S-protein for *in vivo* adherence. As the deletion of S-protein genes is likely lethal [3,10],

more specific mutations could be made. The S-protein gene could be mutated in such as way that predicted surface-exposed regions (i.e. Fig. 3.10A) were altered. Sequences unique to S-protein types a and b in the predicted surface exposed regions would be candidates for mutagenesis, as they might confer unique properties to this S-protein that would improve GIT survival and persistence (highlighted in green in Fig. 3.10A). Alternatively, site-directed replacement of each putative surface exposed region could be performed to elucidate the potential effects of such regions on adherence. To ensure the mutant would express the recombinant protein, replacement of the chromosomal S-protein gene could be conducted as in [16].

Heterologous extracellular expression of S-protein fragments, as done by Martínez et al. [14], Antikainen et al.[1], Åvall-Jääskeläinen et al. [2] and Hynönen et al. [10] could also be performed, although the main drawback lies in trying to simulate an S-layer by using a very different ultrastructure to anchor the S-protein fragments to the cell surface. Although no studies have directly linked the morphology of the S-layer and its ability to adhere to tissue (refer to indirect evidence in [18]), the hydrophobic nature of the outer S-layer surface [20] may suggest initial non-specific interactions between the S-layer and the tissue. Therefore, using specific S-protein sequences in a non-native formation is likely to be less effective, and Martinez et al. [14] did observe this with their engineered protein, as did Antikainen et al. [1] with CbsA fragments expressed by *Lactobacillus casei*. Even if the entire S-protein were expressed in another avian *Lactobacillus* that lacked an S-layer such as *L. johnsonii*, it would be difficult to determine if the S-protein had formed natively and the layer had assembled correctly, attached to the cell wall properly, and that other cell surface components present in the

cell walls of those bacteria had an affect on adherence or S-layer formation. One potential approach could be to replace one of the genes in *L. acidophilus* ATCC 4356 with S-protein type a. As mutation of *slp*A was found to be lethal [3], replacement of *slp*B may be effective. This would allow the mutant *L. acidophilus* to grow *in vitro*. If the S-protein is indeed necessary for *in vivo* survival, it would be expected that recombination to express S-protein type a would occur and adherence to the crop tissue would take place. As *L. acidophilus* ATCC 4356 was found not to persist in the gnotobiotic chicken GIT [15], the recovery of the mutant would suggest that S-protein type a imparts the ability to survive. One potential drawback with this approach may lie in the inability of *L. acidophilus* ATCC 4356 to grow in the chicken GIT if it lacks the necessary enzymes or surface factors to obtain appropriate and sufficient carbon and energy sources in the crop. Our data suggest that S-protein type b, although less conserved among our isolates, is also important for GIT survival. Therefore, the experiments suggested above for S-protein type a could also be performed on S-protein type b.

This research has provided the basis for future analyses of the microflora of the chicken crop and its potential modulation. The crop microflora has not previously been investigated in detail with molecular techniques, nor has the succession of lactobacilli been examined with sufficient specificity to differentiate members of the *L. acidophilus* group. Our data revealed that a poorly studied member of the *L. acidophilus* group, *L. gallinarum*, is present in the crop in high numbers (as determined by selective enumeration at high dilution) and throughout the production period (as determined by PCR-DGGE). These data represent some of the first information gathered on the unique properties of *L. gallinarum*, and the first analysis of the diversity of *L. gallinarum* strains

isolated from their natural environment. Further, we have observed the conservation of two S-protein genes among *L. gallinarum* isolates between different birds and even different flocks. While S-proteins have been found in *L. crispatus* chicken isolates [18] and are predicted to mediate adherence of these isolates in the chicken intestine [1,14,18], ours is the first study investigating the frequency of a particular S-protein among a large and diverse population of lactobacilli obtained from the chicken crop.

The data gathered in this research may alter the approach taken for selection of probiotics to manipulate the microflora of the crop and well-being of the chicken. Rather than selecting probiotics on the basis of adherence of a few strains to *ex vivo* tissue or cell lines, observing the populations of lactobacilli present in the crop of different ages raised under commercial conditions and looking for conserved elements, as our research has done, is likely to indicate crucial factors that are present in many strains of the same species. The conserved S-protein types observed in our *L. gallinarum* isolates suggest they may be essential component for survival and persistence in the GIT, therefore the presence of these proteins on the outer surface of commensal bacteria may be necessary for their persistence and effects on the host. Thus, these S-proteins could be naturally present or engineered into commensal strains to ensure maximum effectiveness.

4.1. References

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Appendix

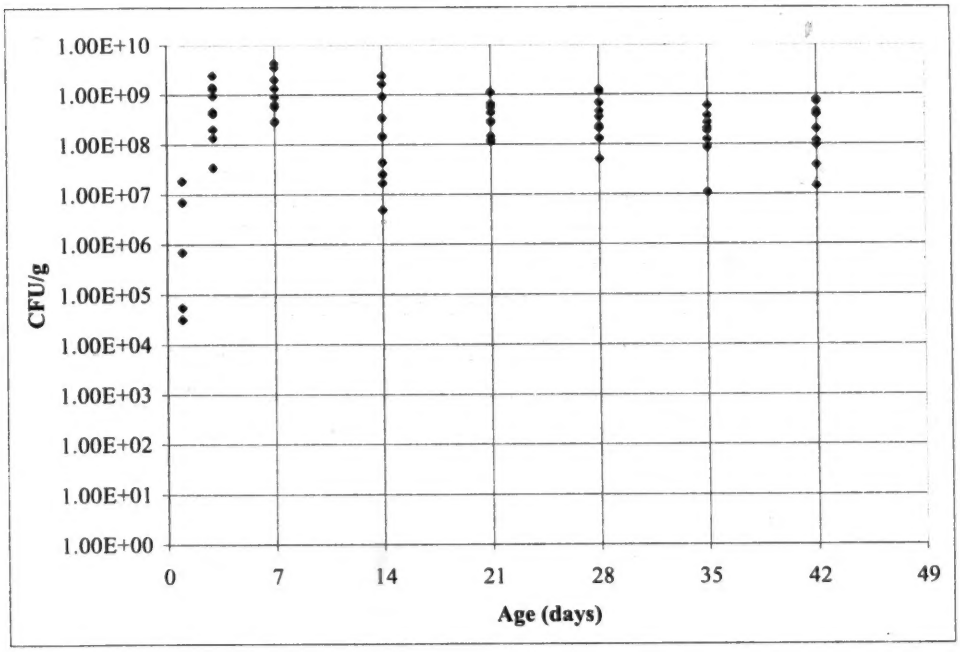

Fig. A.1. CFU/g of lactobacilli isolated from chicken crop homogenates from birds of different ages in the 2nd experiment.

A.

```
Clone D108B    TGCTTTATTAGCTGTTGCTCCTGTCGCTGCTTCAGCCGT---TTCTGTTA  50
Clone D109D    TGCTTTATTAGCTGTTGCTCCTGTTGCTGCTTCTGCTGTAAGCACCGTTA  50
Clone D255A    TGCTTTATTAGCTGTTGCTCCTGTCGCTGCTTCTACTGTAAGCACTGTTA  53
Clone D256B    TGCTTTATTAGCTGTTGCTCCTGTCGCTGCTTCTACTGTAAGTACTGTTA  50
Clone D260#1A  TGCTTTATTAGCTGTTGCTCCTGTTGCTGCTTCTGCTGTAAGCACCGTTA  50
               ************************ ********  * **     * ****

Clone D108B    ACGCTGCTAGTGTTACTTCCGCTACTCAATTAGGTAAGGTACCTACTTTA 100
Clone D109D    ACGCTGCTAGTGTTACTTCCGCTACTCAATTAGGTAAGGTACCTACTTTA 100
Clone D255A    ACGCTGCTAGTGTTACTTCTGCTACCCAATTAGGTAAGGTACCTACTTTA 103
Clone D256B    ATGCTGCTAGTGTTACTTCCGCTACTCAATTAGGTAAGGTACCTACTTTA 100
Clone D260#1A  ACGCTGCTAGTGTTACTTCCGCTACTCAATTAGGTAAGGTACCTACTTTA 100
               * ************************************************

Clone D108B    GCTAATGGTGATGCTGTAAATGTTAAGCCAAATATTTCATTAAACACAGT 150
Clone D109D    GCTAATGGTGATGCTGTAAATGTTAAGCCAAATGTTTCATTAAACACAGT 150
Clone D255A    GCTAATGGTGATGCTGTAAACGTTAAGCCAAATGTTAGCTTGAACACAGT 153
Clone D256B    GCTAATGGTGATGCTGTAAATGTTAAGCCAAATATTTCATTAAACACAGT 150
Clone D260#1A  GCTAATGGTGATGCTGTAAATGTTAAGCCAAATGTTTCATTAAACACAGT 150
               ******************** ************ **   ** ********

Clone D108B    ACATGGTTCAATTAAGGCAGCTATTTCTGTATCATTTGACGCTACTTTTA 200
Clone D109D    ACATGGTTCAATTAAGGCAGCTATTTCTGTATCATTTGACGCTACTTTTA 200
Clone D255A    ACACGGCTCAATTAGCGCTGCTATTTCTGTATCATTTGATGCTACTTTTA 203
Clone D256B    ACATGGTTCAATTAAGGCAGCTATTTCTGTATCATTTGACGCTACTTTTA 200
Clone D260#1A  ACATGGTTCAATTAAGGCAGCTATTTCTGTATCATTTGACGCTACTTTTA 200
               *** ** ********  *  ********************** **********

Clone D108B    ACGGTACTACTGCTACCTCAAACTTTAAGCCTGGATACTCAAACATCCAA 250
Clone D109D    ACGGTACTACTGCTACCTCAAACTTTAAGCCTGGATACTCAAACATCCAA 250
Clone D255A    ACGGTACTACTGCTACCTCAAACTTTAAGCCTGGATACTCAAACATCCAA 253
Clone D256B    ACGGTACTACTGCTACCTCAAACTTTAAGCCTGGATACTCAAACATCCAA 250
Clone D260#1A  ACGGTACCACTGCTACCTCAAACTTTAAGCCTGGATACTCAAACATCCAA 250
               ******* ******************************************

Clone D108B    CTTTTCCACGGTAGCAAGGAAATTACTAATTTACAAGATGTTCATTACCT 300
Clone D109D    CTTTTCCACGGTAGCAAGGAAATTACTAATTTACAAGATGTTCATTACCT 300
Clone D255A    CTTTTCCACGGTAGCAAGGAAATTACTAACTTACAAGATGTTCATTACCA 303
Clone D256B    CTTTTCCACGGTAGCAAGGAAATTACTAATTTACAAGATGTTCATTACCT 300
Clone D260#1A  CTTTTCCACGGTAGCAAGGAAATTACTAATTTACAAGATGTTCATTACCT 300
               ************************************************

Clone D108B    TACTGCAGGTTCAACTTACCGTGTTGTAATGAGCCATGTTGGTTTAAACT 350
Clone D109D    TACTGCAGGTTCAACTTACCATGTTGTAATGAGCCATGTTGGTTTAAACT 350
Clone D255A    TACTGCAGGCTCAACATACCGTGTCGTAATGAGCCATGTTGGTTTGAACT 353
Clone D256B    TACTGCAGGTTCAACTTACC--------------ATGTTGGTTTAAACT 335
Clone D260#1A  TACTGCAGGTTCAACTTACCGTGTTGTAATGAGCCATGTCGGTTTAAACT 350
               *************** ****               **** ***** ****

Clone D108B    TTGGTTCACAAAACGCAAACAAGGAAATCACTTTAACTATGCCTGAAGGC 400
Clone D109D    TTGGTTCACAAAACGCAAACAAGGAAATCACTTTAACTATGCCTGAAGGC 400
Clone D255A    TCGGTTCACAGAATGCTAACAAGGAAATCACTTTGACTATGCCTGAAGGC 403
Clone D256B    TTGGTTCACAAAACGCAAACAAGGAAATCACTTTAACTATGCCTGAAGGC 385
Clone D260#1A  TTGGTTCACAAAACGCAAACAAGGAAATCACTTTAACTATGCCTGAAGGC 400
               * ******** **  * ***************** ***************
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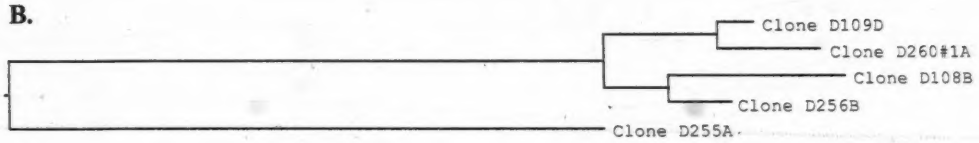
Fig. A.2. Comparison of partial group a S-protein genes cloned form *L. gallinarum* isolates. **A.** ClustalW alignment. **B.** Phylogenetic tree based on DNA sequence comparisons. **C.** Percent identity of S-protein genes.

A.

```
Clone D108B    GATTTCTTCCAACTTGCATCAAACAATACTGTTACAAACTC 441
Clone D109D    GATTTCTTCCAACTTGCATCAAACAATACTGTTACAAACTC 441
Clone D255A    GATTTCTTCCAACTTGCATCAAACAATACTGTTACAAACTC 444
Clone D256B    GATTTCTTCCAACTTGCATCAAACAATACTGTTACAAACTC 426
Clone D260#1A GATTTCTTCCAACTTGCATCAAACAATACTGTTACAAACTC 441
               *******************************************
```

B.



C.

	Clone D108B	Clone D109D	Clone D255A	Clone D256B	Clone D260#1
Clone D108B		97.5	92.5	94.8	97.3
Clone D109D			93.7	95.2	99.3
Clone D255A				90.7	93.4
Clone D256B					94.8
Clone D160#1A					

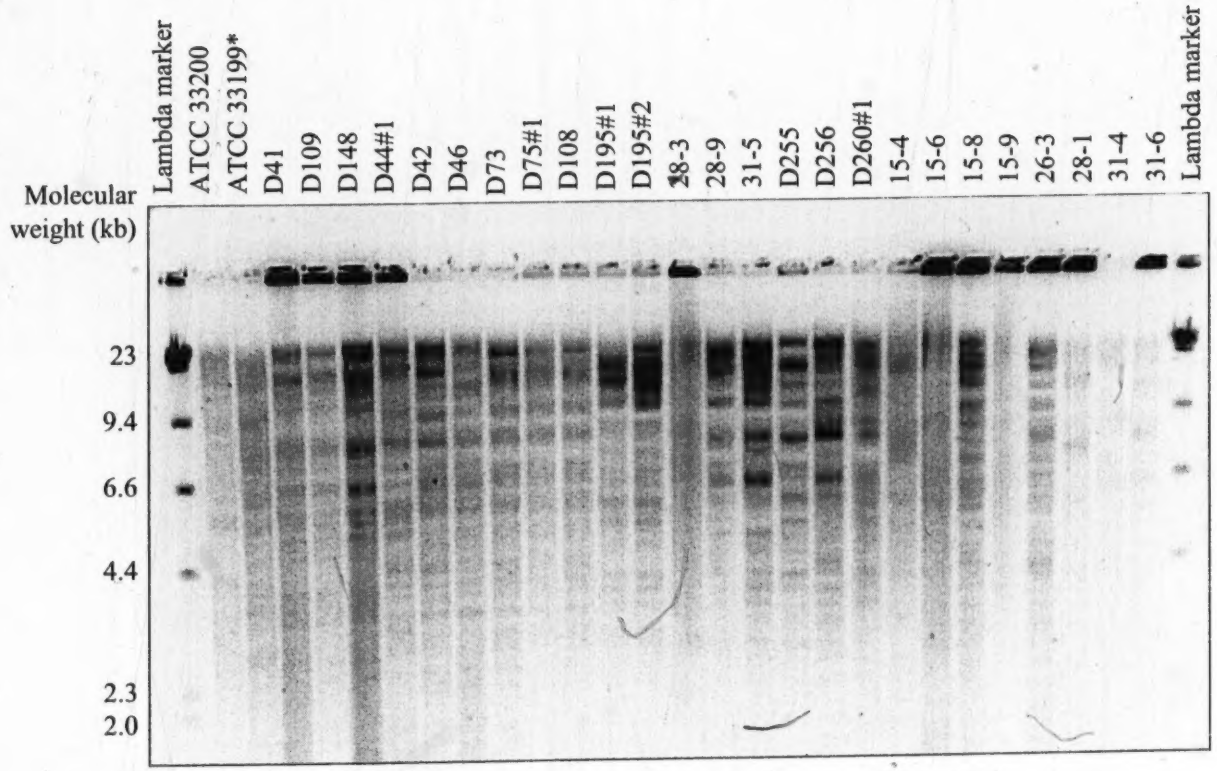


Fig. A.3. *Eco*RI digest of total chromosomal DNA from *L. gallinarum* isolates indicated used for Southern hybridization. "*" indicates positive control.

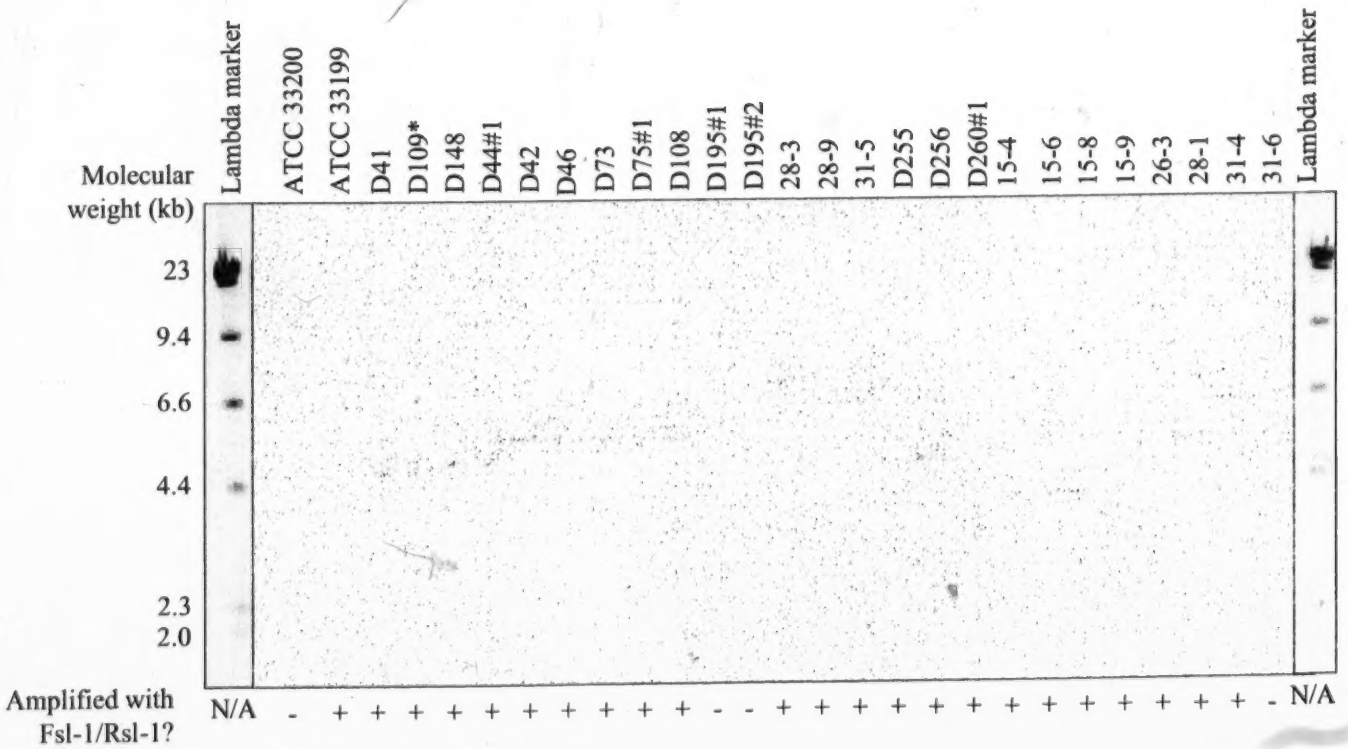


Fig. A.4. Southern hybridization of chromosomal *Eco*RI digests of *L. gallinarum* isolates with S-protein gene type a specific probe. "*" indicates positive control. Text below figure indicates PCR results as presented in Table 3.6. "+" indicates PCR product, "-" indicates no PCR product. N/A - not applicable. Lambda markers derived from Fig. A.3.

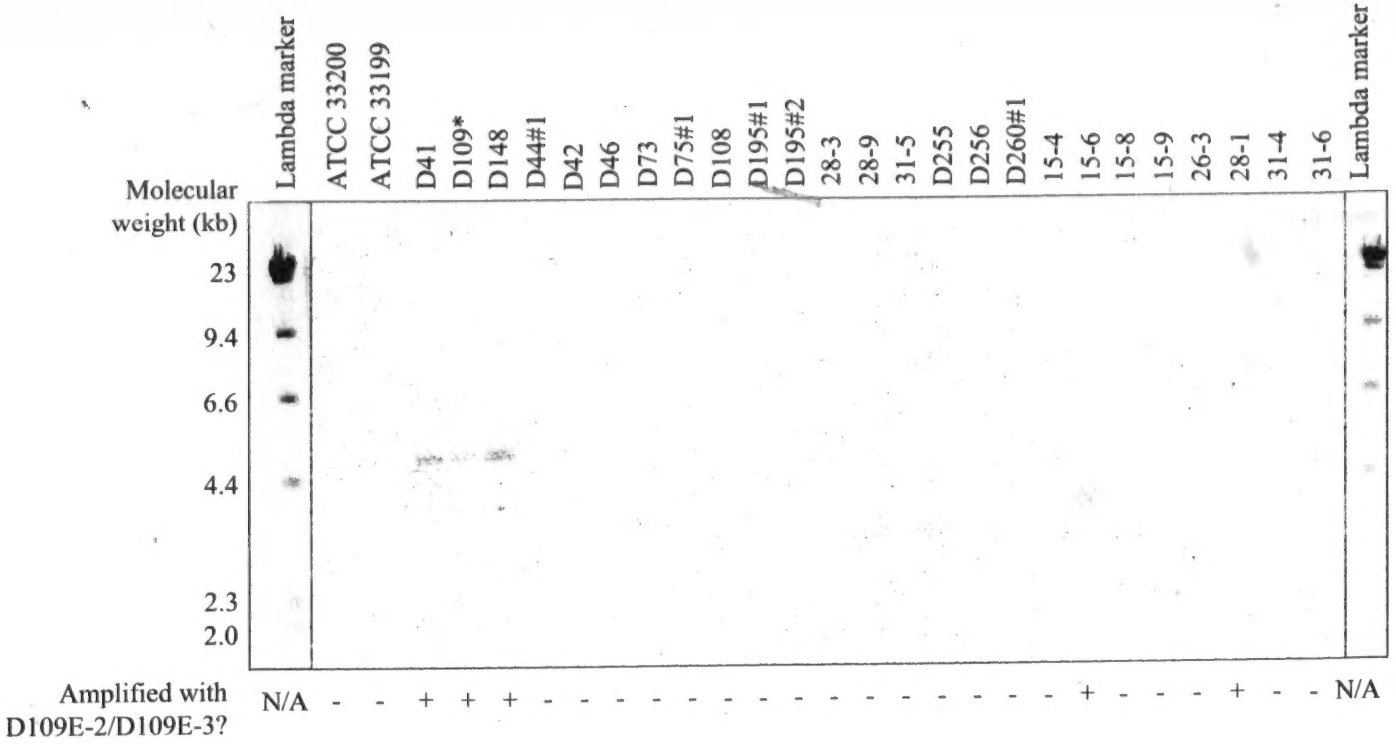


Fig. A.5. Southern hybridization of chromosomal *Eco*RI digests of *L. gallinarum* isolates with S-protein gene type f specific probe. "*" indicates positive control. Text below figure indicates PCR results as presented in Table 3.6. "+" indicates PCR product, "-" indicates no PCR product. N/A - not applicable. Lambda markers derived from Fig. A.3.

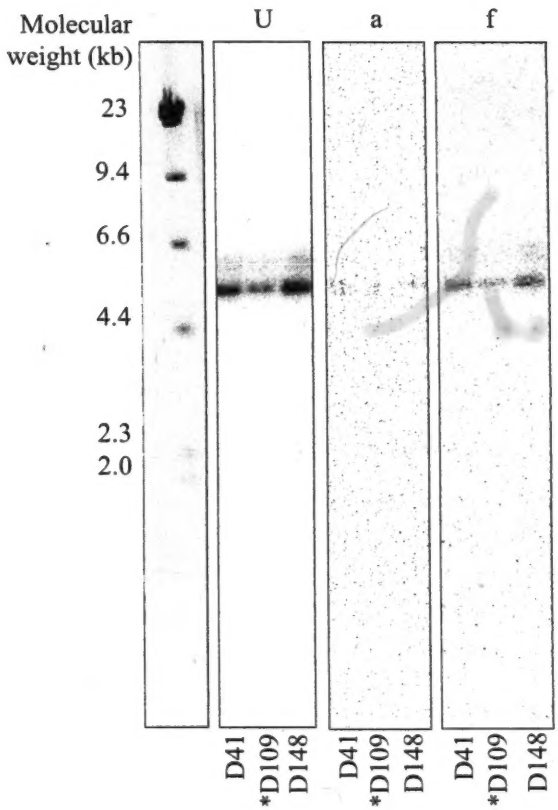


Fig. A.6. Southern hybridization of chromosomal *Eco*RI digests of *L. gallinarum* isolates. Comparison of position of hybridization of U, a and f S-protein probes. "*" indicates positive control. Lambda markers derived from Fig. A.3.

Curriculum Vitae

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Objective

To find employment as technician/research scientist analysing and identifying microbial communities in the gastrointestinal tract and identifying their effects on the host by characterizing specific factors and their mechanisms of action.

Post Secondary Education

Completing Master of Science "Molecular characterization of *Lactobacillus gallinarum* isolates from the broiler chicken crop"	Department of Agricultural, Food and Nutritional Science, University of Alberta. Supervisors: Dr. Gwen. E. Allison and Dr. Gerald W. Tannock	09/2001 - present
Graduated with Bachelor of Science (Honours Microbiology)	Department of Biological Sciences, University of Alberta.	04/2001
Undergraduate research project: isolating and characterising Polyhydroxybutyrate (PHB) depolymerase from PHB granules in *Azotobacter vinelandii*.	Department of Biological Sciences, University of Alberta. Supervisor: Dr. Bill Page.	09/2000-04/2001
Attended 6th annual "Ethics and Scientific Integrity" workshop	University of Alberta (Continuing Medical Education)	01&02 12/2000
International Baccalaureate Diploma Total points: 37/45, received university course credit	Lindsay Thurber Comprehensive High School, Red Deer. Alberta.	1993-1996
Advanced High School Diploma	Lindsay Thurber Comprehensive High School, Red Deer, Alberta.	1993-1996

Professional Skills

Molecular Biology

Skilled in PCR, cloning, PFGE, DGGE, RFLP, RAPD, agarose gel electrophoresis, protein isolation and analysis, and immunological protein detection. Experience analysing microbial communities using culture-independent techniques.

Computers/Technology

Efficient and thorough knowledge of software applications such as Macromedia Flash, Macromedia DreamWeaver, Adobe Photoshop, Adobe Illustrator, Adobe Acrobat, the latest MS Office suites.
Familiar with Mac OS 8 through X.2, and Windows 3.1 through XP, including Windows NT 4 and 2000.

Safety and certification

Certified in Standard Operating Procedures for Poultry according to the AFHE Faculty Animal Policy and Welfare Committee (University of Alberta). Successful completion of Radiation Safety Course (University of Alberta).

Memberships

American Society for Microbiology	11/2002-present
Golden Key Society (also requested to join in November 1997).	11/1999-present
Contributor to Bio-DiTRL, an online, peer-reviewed database of multimedia for instruction and research presentations.	2000

Publications and presentations

Type of presentation	Title	Journal/Conference
Paper	Detection and Identification of *Lactobacillus* Species in the Crop of Broilers of Different Ages Using PCR-Denaturing Gradient Gel Electrophoresis and Amplified Ribosomal DNA Restriction Analysis	Applied and Environmental Microbiology 69:11 (In Press)
Poster	Investigation of the S-layer Protein of the Probiotic Strain *Lactobacillus acidophilus* R0052	Institut Rosell Scientific Exchange, Quebec City, QC September 12-14, 2003
Poster	S-layer variation among *L. gallinarum* strains isolated from the chicken crop	ASM General Meeting (Washington DC), May 18-22, 2003
Poster	Lactobacilli in the Gut of Canadian Broilers	Seventh Symposium on Lactic Acid Bacteria (Egmond aan Zee, The Netherlands) September 2002
Poster	In search of Poly(b-hydroxybutyrate) (PHB) depolymerase in *Azotobacter vinelandii* UWD	Canadian Society for Microbiology, (Waterloo, Ontario) 2001

Professional Experience

TA/marker	**Teaching Assistant, NuFs 480 (Foodborne pathogens)** I helped students in this class with questions, gave several presentations, and assisted with marking.	01/2001- 04/2002
	Marker, NuFs 361 (Food Microbiology) I marked assignments and tests and provided feedback to students.	09/2001- 12/2002
Summer research studentship	**Page lab, Department of Biological Sciences, University of Alberta** I was employed as a summer student to continue research that was started in my undergraduate research project.	05/2001- 08/2001
Communication and Instructional Multimedia	**Digital Teaching Resources Laboratory - University of Alberta** Industrial Internship in the Digital Teaching and Resources Laboratory (DiTRL) at the University of Alberta for the Department of Biological Sciences. I produced and published animations for undergraduate student learning aids on the web using Macromedia Flash 3 and 4. I captured, edited, and published digital video. I created instructional posters and presentations with various software packages. I maintained and updated the lab web page and I assisted students and professors in Biological Sciences with scanning, printing, posters, presentations, publication plates, animations, and video production. I also spent time troubleshooting software conflicts, networking errors, hardware set-up, and performed routine computer maintenance. http://www.biology.ualberta.ca/facilities/multimedia/ Note: Unable to accept employment over summer 1999 with Dr. M. E. Stiles of the University of Alberta and an NSERC summer studentship to take internship in DiTRL. The research involved attempting to understand resistance mechanisms of meat spoilage bacteria to colicins produced by lactic acid bacteria.	05/1999- 09/2000
	Lansdowne Community Church Co-authored a multimedia presentation for Advent 1999. Content contained animation, images, and music and was put together on computer and dubbed to VHS tape.	12/1999
	Lansdowne Community Church Design, production and photocopying of church bulletins made in MS Publisher 98.	041/1999- 06/2000
Instruction	**Self employed** High school biology and chemistry tutor to Edmonton high school student.	09/1999- 03/2000

	Volunteer English Teacher – Michener Park I taught basic and intermediate English to immigrants and international students.	01/1999- 12/1999
	Volunteer English Teacher – Student Volunteer Campus Community I taught advanced grammar to 5 Cantonese students.	05/1999- 08/1999
Warehouse	**Head Shipper – CompuSmart West Edmonton** I carefully packaged customer products and worked with shipping companies to arrange for pick-up and delivery of products. I also aided the shipping and receiving department by working overtime to ensure the inventory and hold reports of the store were correct.	05/1998- 08/1998
Customer Service	**Café cook – CompuSmart Keyboard Café** I acted as hostess, bus-girl, cashier, and cook. While employer was on vacation, I was responsible for product ordering, food preparation, cleanup, and café specials.	05/1997- 08/1997
Service	**Lansdowne Community Church Music Team** I have served as leader, administrator, and singer.	1997- 2000
	Services for Students with Disabilities I have volunteered to take notes for two vision-impaired students at the University of Alberta.	1997- 1998
	Capital Care Grandview Retirement Home I helped approximately 20 residents each week for "Friday Movie Night" by taking them to and from the viewing room and serving them popcorn and juice during the movies.	1997- 1998
	Volunteer – Red Deer Regional Hospital I helped in several different departments including flower delivery, café, patient care, and gift shop.	1993- 1995

Scholarships

Scholarship	Awarded by:	Date held
NSERC PGSA	NSERC	2001-2003
Hazel McIntyre Summer Research Award	University of Alberta	2002
Walter John Memorial Scholarship	University of Alberta	2001-2003
NSERC Summer Studentship	NSERC	2001
Chancellor's Citation Scholarship	University of Alberta	1996-2001
Bill Paranchych Memorial Scholarship	University of Alberta	2000-2001
Faculty of Science Undergraduate Scholarship	Faculty of Science, University of Alberta	1996, 1997, 1999
Louise McKinney Post-Secondary Scholarship	Province of Alberta	1997
Faculty of Science Entrance Scholarship	Faculty of Science, University of Alberta	1996
International Baccalaureate Scholarship	University of Alberta	1996
Academic Excellence Entrance Scholarship	University of Alberta	1996
Parkland Savings and Credit Union Scholarship	Lindsay Thurber High School, Red Deer, AB.	1996
Alexander Rutherford Scholarship	Lindsay Thurber High School, Red Deer, AB.	1996

References

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